Biochemical characteristics and plant tissue localization of plant growth-promoting bacteria isolated from sugar beet (*Beta vulgaris* L.)

甜菜から分離された PGPB の生化学特性と植物組織における局在

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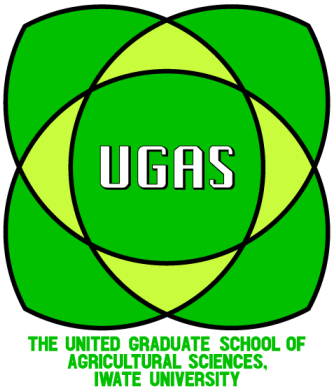

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Abstract

Utilization of plant growth-promoting bacteria colonizing roots is environmentally friendly technology instead of using chemicals in agriculture, and understanding of the effects of their colonization modes in promoting plant growth is important for sustainable agriculture. In this study, the six potential plant growth-promoting bacteria (*Rhizobium* sp. HRRK005, *Polaromonas* sp. HRRK103, *Variovorax* sp. HRRK170, *Mesorhizobium* sp. HRRK190, *Streptomyces* sp. HRTK192, and *Novosphingobium* sp. HRRK193) isolated from sugar beet (*Beta vulgaris* L.) were screened as a bioinoculant and selected strain's effectiveness was evaluated using vegetables seedlings. The results showed that *Variovorax* sp. HRRK170 had the highest potential for plant growth promotion, given its ability to produce multiple plant growth substances (siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, biofilm and indole-3-acetic acid (IAA) and active growth in a wider range of temperatures (10–30 ºC) and pH (5.0–10.0). HRRK170 cells colonized in all vegetables. But significant growth promotion occurred only in two vegetables (Chinese cabbage and green pepper). When the density of infected cells on seedlings was determined based on GUS-staining intensity, results showed that optimally infected cells led significant increment in plant growth, as observed in green pepper and Chinese cabbage. Therefore, it was considered that the optimum cell density may reflect the beneficial interaction between plants and HRRK170.

Also, salt (NaCl) and drought stress (PEG) stress mitigating effect of HRRK170 was investigated using two cultivars of Chinese cabbage (Haregi 85 and Kigokoro 85). HRRK170 mitigated salt stress with efficient plant tissue localization and increased growth-promoting compounds production in both two cultivars and drought stress

mitigated in cv. Kigokoro 85, which seemed sensitive to stresses. Findings of this study suggested that HRRK170 could function as a plant growth promoter with an optimum cell density for efficient use and potential plant salt and drought stresses mitigating bacteria.

Abstract in Japanese

根にコロニーを形成する植物成長促進細菌の利用は、農業において化学物質の代わりとなる環境に優しい技術である。そして、植物成長の促進におけるそれらのコロニー形成の知見は、持続可能な農業にとって重要である。本研究では、テンサイ（*Beta vulgaris* L.）から単離した 6 株の植物成長促進効果を持つ可能性のある細菌（*Rhizobium* sp. HRRK005、*Polaromonas* sp. HRRK103、*Variovorax* sp. HRRK170、*Mesorhizobium* sp. HRRK190、*Streptomyces* sp. HRTK192、および *Novosphingobium* sp. HRRK193）を微生物資材としてスクリーニングし、選択した株の有効性を野菜実生を用いて評価した。結果は、HRRK170 が複数の植物成長物質（シデロフォア、1-アミノシクロプロパン-1-カルボン酸（ACC）デアミナーゼ、バイオフィルムおよびインドール-3-酢酸）を産生する能力を持ち、より広い温度範囲（10～30℃）および pH（5.0～10.0）で増殖したことから、最も高い植物成長促進の可能性があることを示した。HRRK170 細胞はすべての野菜実生の根の表皮に定着したが、有意な成長促進は 2 つの野菜（ハクサイとピーマン）でのみ確認された。植物体上の感染細胞の密度は GUS 染色強度に基づいて決定され、最適感染細胞密度においてピーマンとハクサイで植物成長の有意な増加をもたらすことを示した。したがって、最適細胞密度は植物と HRRK170 株との間の有益な相互作用を反映していると考えられた。また、ハクサイの 2 品種（Haregi 85 と Kigokoro 85）を用いて、HRRK170 株の食塩（NaCl）および乾燥（PEG）ストレス軽減効果を調べた。HRRK170 株は、植物組織局在化および成長促進物質の増加により、塩ストレスの軽減は 2 つの栽培品種の両方でみられ、乾燥ストレスの軽減効果は Kigokoro

85 においてのみ確認された。この研究の知見は、HRRK170 が最適細胞密度の使用によって植物成長促進剤として機能するだけでなく、植物への塩および乾燥ストレス軽減剤として機能することを示唆した。

CHAPTER 1.

Introduction and literature review

1.1. Plant growth-promoting bacteria

Plants have an ability to convert light energy to chemical energy, produce nutrients and continually release it as a rhizodeposits [water-soluble exudates (eg., sugars, amino acids, organic acids and enzymes) and water-insoluble materials (e.g., mucilage and sloughed cells)] through their roots. Organic compounds released as rhizodeposits support the growth of microorganism in soil (Merbach *et al.,* 1999), particularly, microbes which accounts for 95% of colonizing microorganisms (Glick, 2012; Neumann, 2007), and important determinant of rhizosphere microorganisms community structure (Neumann, 2007). Microbes that are able to colonize on plant tissue efficiently and deliver multiple positive effects to plant growth and development (e.g., increased seed emergency, plant resilience and crop yield) are termed as a plant growth promoting bacteria (PGPB) (Ping & Boland, 2004). These bacteria include bacteria that involve formation of nodule on roots, free living cells (rhizsphere bacteria or rhizobacteria) as well as endophytes which can be found inside plant tissues (Glick, 2012).

On the other hand, intensive agriculture became dependent on excessive use of chemical fertilizers due to food demand of rapidly increasing human population. These chemicals may cause potential hazard to public health and environment (de Souza, 2015; Olanrewaju, Glick, & Babalola, 2017; Vessey, 2003). Residues of agricultural chemicals cause loss of biodiversity and is disturbance in nutrients cycles and biological processes that damage soil structure and disable long - standing agricultural productivity.

Additionally, climate change will continue to affect as disrupting extreme weather conditions on food production (Vujanovic & Germida, 2017). Hence, the 21st century is a challenging era for crop production. Therefore, PGPB is drawing attention as a research target for their contribution to plant growth enhancement and abiotic stress tolerance or biocontrol activity and maintenance of natural fertility of soil (Toyota, 2013). Indeed, microbial bioinoculants may simultaneously reduce the use of chemicals, while enhance crop productivity (Belimov *et al.*, 2009; van Veen, van Overbeek, & van Elsas, 1997).

PGPB are commercialized as biofertilizers (bioinoculant) and biocontrol agents or biopesticides. Initially, *Rhizobium* inoculants had been developed as commercial products. Currently, one or more species of *Agrobacterium sp*, *Azospirilum* sp., *Bacillus* sp., *Azotobacter* sp., *Burkholderia* sp., *Delfitia* sp., *Paenobacillu* sp., *Pseudomonads* sp., *Serratia* sp., *Streptimyces* sp. and *Rhizobia* sp. have been commercialized worldwide (Glick, 2012). Development of PGPB biofertilizers is increasing throughout worldwide, due to damages to nature caused by inadvisable use of agricultural chemicals and the advanced knowledge about plant microbe interaction (Timmusk, Behers, Muthoni, Muraya, & Aronsson, 2017). However, only 5% of total fertilizers in agricultural practice is composed of PGPB inoculants (Glick, 2012; Timmusk *et al*., 2017), since microbes used for these purposes are very specific and several steps of specialized screening and experimenting have to be performed to reach final the level of production (Ahmad *et al.*, 2018).

Better understanding about the efficient action or role of PGPB to the host plant growth can contribute to developing PGPB based strategies that can be used successfully for sustainable agriculture.

1.2. Mechanisms of plant growth promotion by bacteria

Yield improvement by inoculation of PGPB mainly occurs due to increased nutrient uptake, stimulation of hormones and inhibition activity of pathogens. Benefits that plants gain from the interacting with PGPB are reflected by increase of germination rates, shoot and root weights, root growth, improved chlorophyll content, leaf area, magnesium content, content of nitrogen and protein, hydraulic activity, tolerance to stresses (Lucy, Reed, & Glick, 2004). Therefore, the beneficial interaction between plant and bacteria is one of the key factors determining plant health, productivity, and soil fertility (de Souza, 2015; Toyota, 2013; Vessey, 2003).

PGPB can have one or multiple traits to facilitate plants growth and act at different stages via several mechanisms, either simultaneously or sequentially (Shahzad, Arshad, Khalid, & Mehboob, 2008). There are contradictory reports about effectiveness of PGPB on host plant growth under various soil conditions. For example, positive effect of *Azospirillium* only in nitrogen limited soil had been reported by Dobbelaere (Dobbelaere *et al.*, 2001), while this species could improve yield by addition to nitrogen fertlized soil (Okon & Labandera-Gonzalez, 1994). Therefore, comparative studies on interaction among different plants or strains are necessary. Generally, mechanisms that PGPB employ to promote plant growth are classified as direct and indirect mechanisms. Additionally, competitive colonization capacity is crucial for both inoculants as biofertilizers, biopesticides, phytostimulators, and bioremediators (B. J. Lugtenberg, Dekkers, & Bloemberg, 2001).

Direct mechanisms:

Improved nutrient acquisition: There are at least 14 elements that are essential for plant growth. Essential nutrients such as nitrogen and phosphorus are added to the soil as

fertilizer and excessive part in soil cause pollution. In this regard, nitrogen fixing bacteria such as *Rhizobia* and *Azospirillium* sp. strains were widely studied as it can fix nitrogen from atmosphere and make it available for plant absorption. Also, free living bacteria provide small amount of nitrogen to host plants as it is required (James & Olivares, 1998). Some microbes solubilize phosphorus and make it available for plant uptake, although it is mostly present in soil in adequate amount. Similarly, iron which abundantly exists in nature is sparingly soluble, but its is highly needy for the many physiological processes of plant (Guerinot & Yi, 1994). Iron deficiency causes nutritional disorder in crops (Forni, Duca, & Glick, 2017). Hence, the capacity to produce siderophore is a beneficial trait of PGPB as it increases iron uptake by the host plant (Ahmad *et al.*, 2018; Olanrewaju *et al.*, 2017). On the other hand, stress can be caused by low Zn availability in soil, especially for cereals. Zn-mobilizing phytosiderophore releasing PGPB can effectively improve growth of plant in Zn limited condition (B. Singh, Natesan, Singh, & Usha, 2005).

Phytostimulation: Microbial phytohormone (auxin, abscisic acid, cytokinins and gibberellins) production is also an important trait of PGPB, because they can promote plant growth both under normal or stressed conditions. PGPB produce phytohormones required by plant and save metabolic energy of plant for growth and reproduction (cell enlargement, seed germination, root formation and stem elongation) (Ahmad *et al.*, 2018). Ethylene is an important hormone for plant that determines plant maturity and low level of this homrone is required during normal growth and development. Although, it is a stress hormone that may reduce plant growth under stressful condition (reduced root, shoot lenght and stem diameter) due to high concentration in tissue (Ahmad *et al.*, 2018). Therefore, bacterial ACC-deaminase activity that breaks ACC into α-ketobutirate, which is the immidiate precursor of ethylene, is important trait to mitigate stress effect in plant (Belimov *et al.*, 2009; Glick, 2005, 2012). ACC-deaminase activity of several bacterial

strains had been successfully studied under biotic and abiotic stresses (Mayak, Tirosh, & Glick, 2004).

Indirect mechanisms:

Suppression of growth of pathogens and decreased damage by infection are usually attributed to the presence of PGPB, which is indirect mechanisms of PGPB (Glick, 2012). Bacterial metabolites such as viscosinamide, 2,4-diacetyphlaroglucinol, phenazines, pyoluteorinand, tensin, and pyrrolnitrin are antifungal compounds. Additionally, antifungal metabolite hydrogen cynide works synergistically with antibiotics and improves efficiency (Bhardwaj, Ansari, Sahoo, & Tuteja, 2014; Compant, Duffy, Nowak, Clément, & Barka, 2005; Glick, 2012; Raaijmakers, Vlami, & de Souza, 2002). Also, lytic enzyme activities (lipases, proteases, glucanases and chitinases) of PGPB suppress growth of fungal phytopatogens (Ahmad *et al.*, 2018), and especially, chitinolytic bacteria are expected to be efficient to suppress fungal growth, since fungi contain chitin as the main component of their cell wall (Toyota, 2013).

Additionally, siderophores produced by bacteria limit the fungal uptake of iron, and therefore suppress their growth (Glick, 2012). Moreover, many plants can use iron-bond siderophore (Y. Wang, Brown, Crowley, & Szaniszlo, 1993), while pathogenic microorganisms do not (Ahmad *et al.*, 2018). Another important trait of PGPB is inhibition to phytopathogens attack by triggering defensiveness termed as induced systematic resistance (ISR) in plant. ISR lead several defensive actions including developmental change, symptom expression and cell wall enforcement (Berg, 2009). Biocontrol activity of PGPB effects on pathogens locally, or the near surface of plant tissue by producing secondary products such as antibiotics, siderophores, and a several types of enzymes and can also function as competitor on colonization sites of pathogens (Carlos A *et al.*, 2007). Some bacterial produce antifreeze proteins that regulate ice

crystallization outside of the bacterium and it protects plant cell from lethargic damage during freezing winter temperature. Also some of them produces trehalose (disaccharide) which protects plant from salt, drought and extreme temperature (Glick, 2012).

1.3. PGPB isolated from sugar beet

Sugar beet is one of the most important crops as sucrose source (Cooke & Scott, 1993). In Japan, sugar beet is the main source of sucrose (approx. 75%) and an important rotational crop in Hokkaido, where it only grows (Hongo & Niwa, 2012). With high yield and well adaptation in local environment, bacterial and fungal communities of sugar beet attracted as a study subject as a study subject, due to an assumption that the higher yield of sugar beet compared with other temperate crops might be contributed by high affinity of PGPB associated with it. Okazaki *et al*., (2014) reported that bacterial community, isolated from root of sugar beet 'Rycka' grown in Hokkaido, Japan, was dominated by *Alphaproteobacteria* (46.7–64.8%) followed by *Actinobacteria* (9.8-16.6%) and *Betaproteobacteria* (6.8-20.7%). Another study by this team concluded that genes involved in β-1,3-glucanase activity, methanol utilization, siderophore production, and ACC deaminase were detected as a PGP traits of bacterial community of sugar beet (Tsurumaru *et al*., 2015).

Sugar beet associated bacterial community was also had been characterized in several studies. Previously, strong antagonisms against phytopathogens were observed in bacterial and fungal communities isolated from sugar beet (Bhattacharya, Chanda, & Barik, 2009). Na Zhou *et al.,* (2017) reported that halophytic PGPB isolated from sugar beet promoted growth of sugar beet when it was planted under salt stress (Zhou *et al.,* 2017). Additionally, high diversity of bacterial microbiome had been recorded from sugar beet (Shi, Yang, Zhang, Sun, & Lou, 2014). This plant is comparatively tolerant to local

environmental weather condition among other local crops and abundant interaction with bacterial strains makes this plant as a study object as a source of beneficial microbes.

1.4. Importance of PGPB on vegetables growth

Vegetables are an important part of the human dietary and rich in many essential vitamins, carbohydrates, salts and proteins. However, soil fertility loses or structure damage and environmental deterioration caused by extended use of chemical fertilizers lead reduction in vegetables production (Jaiswal, Verma, Krishna, Gaurav, & Yadav, 2019; Zaidi *et al.,* 2017). Additionally, many vegetables farmers gradually avoided to use of agricultural chemicals, but aimed to substitute it by organic agricultural systems vegetables. Beneficial bacterial inoculants play in important roles in organic farming as a component by improving nutrient uptake and mineralization (Zaidi & Khan, 2017). PGPB inoculant keeps freshness of vegetables (increased the antioxidant activity, the total phenolic compounds and chlorophyll) and nutritional value and properties (taste, texture and safety) (Alori & Babalola, 2018).

Furthermore, vegetables are more sensitive than other horticultural plants to abiotic stresses and each vegetable species needs a specific condition (temperature, humidity, light intensity, soil moisture and wind) for optimal growth (Zaidi & Khan, 2017). Previously, various PGP bacterial positive effect on vegetable growth was observed due to improved root length, stress tolerance (especially adaptation during transplantation), seed aging and germination (Carlos A *et al.,* 2007). Thus, the use of PGPB based bioinoculant is supported as a feasible alternative to chemical fertilizers in agricultural practice of vegetables.

1.5. Objectives of the study

This study aimed to identify the highest potential bacterial strain from bacterial isolates, which is isolated from sugar beet as an inoculant with regard to its beneficial traits of PGPB and evaluate the selected strain's effectiveness.

Objectives were as follows:

1) To screen bacterial isolates as a bioinoculant and select the highest potential PGPB strain.
2) To elucidate selected strain's growth promoting effect as bioinoculant using vegetable seedlings.
3) To elucidate the selected strain's role under stressed conditions as a plant stress mitigating bacteria.

CHAPTER 2.

Characteristics of PGPB isolates from sugar beet as a bioinoculant

2.1. Introduction

Plant harbors variable bacterial communities and their interactions determine plant health and productivity. Determining whether bacteria would act as plant growth promoter or inhibit growth is important step of the study to develop PGPB based inoculant (Glick, 2012). There is the evidence that local environmental condition effect on formation of plant microbe interaction (Farrar, Bryant, & Cope-Selby, 2014). Moreover, plants with high yield form an important relationship with microbes, in order to acquire essential macro and micronutrients through the interaction (Miransari, 2013). Furthermore, native bacterial strain performs better as growth promoter than new strain introduced from other location, since the interaction between plant and microbe depends on various environmental parameters and native PGPB are suited in that environment (Bashan, de-Bashan, Prabhu, & Hernandez, 2014).

In the Tokachi area, Hokkaido, Japan, volcanic ash causes soil acidity and aluminum toxicity (Shoji & Takahashi, 2002), which reduce crop yield by restricting root systems and the uptake of water and nutrients (Samac & Tesfaye, 2003). However, yields of sugar beet, which are important as both a source of sugar and in crop rotation, are persistently high, presumably because of the interactions with useful microorganisms such as PGPB (Tsurumaru *et al.*, 2015). In a previous study, bacterial strains having a high affinity with the sugar beet (*Beta vulgaris* L.) were examined for their plant growth-

promotive abilities (Kenkyuseika, vol. 539. 2015. Tsukuba Office, Agriculture, Forestry and Fisheries Research Council Secretariat, Japan). In this study, six potential PGPB strains were used for selection of potential bioinoculant. In order to identify the highest potential strain, these strains were screened for tolerance against pH and temperature stresses since effectiveness of plant growth-promoting bioinoculants likely depends on bacterial survivability to sustain beneficial interactions with the host plant (van Veen *et al.*, 1997). Afterwards, six PGPB strains were biochemically characterized for production of plant growth promoting substances and enzyme activities. The efficiency of bioinoculant is determined by bacterial capacity to colonize plant root (Danhorn & Fuqua, 2007; B. Lugtenberg & Kamilova, 2009). Therefore, the selected strain was examined by capacity to colonize plant tissue. Based on results, *Variovorax* sp. HRRK170 was selected as the highest potential bioinoculant as it showed multiple beneficial traits as a bioinoculant that led significant positive influence on growth of sugar beet.

2.2. Materials and methods

2.2.1. Bacterial strains and growth conditions

Bacterial strains with high affinity for sugar beet roots (*Beta vulgaris* L.) were previously studied for their plant growth promotion (Kenkyuseika, vol. 539. 2015. Tsukuba Office, Agriculture, Forestry and Fisheries Research Council Secretariat, Japan). Among them, six potential plant growth-promoting bacterial strains, *Rhizobium* sp. HRRK005 (NITE P-01604), *Polaromonas* sp. HRRK103 (NITE P-01607), *Variovorax* sp. HRRK170 (NITE P-01608), *Mesorhizobium* sp. HRRK190 (NITE P-01609), *Streptomyces* sp. HRTK192 (NITE P-01614), and *Novosphingobium* sp. HRRK193 (NITE P-01610) were used in this study. All strains were grown in R2A medium (BD, Sparks, MD, USA), and preculture was adjusted based on OD (OD_{600} = 0.1) to initiate culture.

2.2.2. Bacterial pH and temperature stress tolerance

To determine growth of six strains at different temperatures and pH values, a portion of preculture, grown aerobically in R2A medium at 30 °C, was transferred into the same fresh medium and grown at 10, 20, 30, and 40 °C or into the fresh medium adjusted to pH 4.0, 5.0, 6.0, 8.0, 9.0, and 10.0, and grown at 30 °C. Growth was monitored at 660 nm by using a biophotorecorder (TVS062CA; Advantec Co., Tokyo, Japan).

2.2.3. Evaluation of biochemical characteristics

Production of indole-3-acetic acid (IAA) was quantified by high performance liquid chromatography (HPLC) analysis using ethyl acetate extraction method (Tien, Gaskins, & Hubbell, 1979). In brief, bacterial cells were grown in 200 mL R2A broth containing 2 mM L-tryptophan at 30 °C and 130 rpm for 7 days. The supernatants were

collected by centrifuging at 9,800 x g for 3 min, and after pH was adjusted to 2.8 by using 1N HCl, IAA was extracted with 200 mL ethyl acetate, twice. Then, the ethyl acetate fraction was evaporated under vacuum and the residue was dissolved using 1 mL methanol. Samples were filtered through a membrane filter (pore size, 0.2 µm) prior to HPLC analysis. IAA production was detected by HPLC (Tosoh Co., Tokyo, Japan) at 254 nm using a column of TSK gel ODS-100V (5µm, 4.6×150 mm, Tosoh Co., Tokyo, Japan). Isocratic separations were performed using a 0.1% H_3PO_4 in acetonitrile:water (60:40 v/v) mobile phase with a flow rate of 1 ml/min. IAA production levels were expressed as micrograms of IAA per mg dry weight of cells.

Siderophore production was evaluated using chrome azurol S shuttle assay (Schwyn & Neilands, 1987) with minor modifications. In brief, bacterial precultures (50 µL) were inoculated into 5 mL of R2A broth and incubated at 30 °C and 130 rpm for 7 days. After centrifugation, 900 µL of the supernatant (n = 4) was incubated for 20 min with 100 µL chrome azurol S and 10 µL of 400 mM sulfosalicylic acid, the shuttle agent. As described above, the R2A broth (900 µL) reacted with chrome azurol S and was used as a reference. Siderophore production levels were expressed as the ratio of the final absorbance value of the sample [absorbance of the reference solution at OD_{630} minus absorbance of sample at OD_{630}] against the reference solution.

Biofilm production was determined by microtiter plate assay (Yuttavanichakul *et al.*, 2012), in which bacterial precultures adjusted to the same initial OD (OD_{600} = 0.1) were incubated statically at 20 °C for 48 h in 96-well polystyrene microplates. After removing the culture, the wells were air-dried and stained for 45 min with 200 µL of 1% crystal violet (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). After dissolving in 95% ethanol, the product was assayed at 595 nm using a microplate reader (iMark, Bio-Rad Laboratories, USA).

The ACC-deaminase activity was determined by measuring the amounts of α-ketobutyrate produced by the reaction (Penrose & Glick, 2003). Briefly, bacterial cells were grown in R2A medium at 30 °C and 130 rpm for 48 h, and after centrifugation, washed with 5 mL of DF salts minimal medium (Dworkin & Foster, 1958). Then, the cells were resuspended in 7.5 mL DF salts minimal medium containing 3 mM ACC and grown for 24 h to induce ACC deaminase activity. After the cells were washed with 1 mL of 0.1 M Tris-HCl (pH 7.6) and resuspended in 600 µL of 0.1 M Tris-HCl (pH 8.5), they were mixed with 30 µL toluene. Subsequently, the suspension (200 µL) was supplemented with 20 µL of 0.5 M ACC and 1 mL of 0.56 M HCl and further acidified with 800 µL of 0.56 M HCl, incubated at 30 °C for 15 min. Next, 300 µL of 0.2% 2,4-dinitrophenylhydrazine was added to the mixture and incubated at 30 °C for 30 min. The activity was expressed by the formation of α-ketobutyrate (nanomole per mg of cell for 1 h at given conditions).

β-1,3-glucanase activity was evaluated by measuring the amounts of reducing sugar released from laminarin as described by Singh *et l.*, (1999). Briefly, bacterial cultures grown for 48 h in 1% colloidal chitin (Sigma-Aldrich, St. Louis, MI, USA) at 30 °C and 130 rpm were centrifuged at 4 °C and 9800 x *g*, and 1 mL of the resulting supernatant was reacted at 40 °C for 1 h with 0.1 mL of 2% laminarin in 0.2 M acetate buffer (pH 5.4). Development of brown color was quantified at 530 nm. A standard curve was plotted using glucose, and one unit of the activity was defined as the amount of enzyme that liberates one micromole of glucose per hour at the indicated conditions.

Bacterial chitinase activity was measured on R2A agar plates supplemented with colloidal chitin (O'Brien & Colwell, 1987), while cellulase activity was quantified on LB plates with carboxymethyl cellulose (Crabbe, Campbell, Thompson, Walz, & Schultz, 1994). Lipase activity was assessed on R2A agar plates with Tween 20 (FUJIFILM Wako

Pure Chemical Co., Osaka, Japan) as substrate (Bhattacharya *et al.*, 2009). These enzyme activities were quantified based on the diameter of halo zones formed around colonies after bacterial precultures (5 µL) were spotted on plates and incubated at 30 °C for 7 days.

2.2.1. *Detection of tissue localization of HRRK170 in sugar beet seedlings*

To observe plant tissue localization of HRRK170 cells, a reporter gene, *gusA*, was introduced into the cells to construct GUS-labeled cells using methods described previously (Simon, 1984). Briefly, *Escherichia coli* S17-1 harboring the plasmid pmTn*5*SS*gusA20* (donor) and HRRK170 (recipient) cells were mixed, and after centrifugation, cell pellets were resuspended in 50 µL of 0.85% NaCl. Mating was performed at 30 °C for 2 days on a membrane filter (mixed cellulose ester; pore size 0.45 µm, Advantec Co., Tokyo, Japan) placed on R2A agar plate containing spectinomycin (100 µg mL^{-1}), streptomycin (40 µg mL^{-1}), and tetracycline (15 µg mL^{-1}). Transformed HRRK170 cells were confirmed to promote plant growth to a similar extent as the wild type.

Sterilized sugar beet seeds were sown on 1.5% agar plates and covered with aluminum foil prior to germination. Seedlings were then transferred to 0.3% agar plates containing a 2000-fold dilution of HYPONeX 6-10-5 (HYPONeX, Osaka, Japan), and after inoculation with 100 µL of GUS-labeled HRRK170 cells (8.5×10^7 CFU mL^{-1}) per seedling, grown in a growth chamber (BiOTRON; NK system, Osaka, Japan) under the same conditions described above. HRRK170 cells localized in seedlings were stained in a GUS-staining solution containing 16 mL of 125 mM sodium phosphate buffer (pH 7.0), 80 µL of 0.5 M Na_2EDTA (pH 8.0), 200 µL of 2% 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt, 80 µL of 10% sodium dodecyl sulfate, and 23.6 mL of distilled water. The staining was performed in de-aired conditions for 90 min and

then left at 30 °C for 2 h prior to use. Microscopic observation of the localized HRRK170 cells in seedlings was conducted using a stereomicroscope (SZX16, Olympus Co., Tokyo, Japan).

To observe localization of cells in root by scanning electron microscopy, seedling's root was cut and fixed in 2% Glutaraldehyde (GA) solution for 2 h and treated with 50%, 70%, 80%, 90% and 99% ethanol for 15 min respectively. Afterwards, samples were dehydrated by 99% tert butyl alcohol, lyophilized and coated with gold using an MSP-mini magnetron sputter prior imaging on a SEM (Miniscope TM3030). In parallel, inoculated seedlings were treated with LIVE/DEAD *Bac*Light bacterial Viability Kit (Invitrogen, CA, USA) according to the instruction and observed under fluorescence microscope (BZ-X700, Keyence Co., Osaka, Japan). All experiments regarding tissue localization of HRRK170 cells were performed at 7 days post inoculation.

2.2.2. *Inoculation test of HRRK170 and plant growth promotion*

Autoclaved PotAce N potting soil (Katakura & Co-op Agri, Tokyo, Japan) was used to assess the plant growth. Sugar beet seeds (*Beta vulgaris* L. cv. Rycka) were provided by the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Hokkaido, Japan.

Seeds were left under running tap water for 24 h, sterilized with 70% ethanol for 1 min, 0.5% sodium hypochlorite containing 0.1% Tween 20 for 15 min, and then rinsed with sterilized distilled water for 15 min (three times). Sterilized seeds were sown on plug trays containing approximately 100 g autoclaved soil (at 121ºC for 20 min) and inoculated with 1 ml of 8.5×10^7 CFU mL^{-1} bacterial suspension as an inoculant and its dilutions (from 8.5×10^4 CFU mL^{-1} to 8.5×10^6 CFU mL^{-1}) per 4 seeds. Seeds were covered with aluminum foil for 7 days, and then grown for four weeks in a growth chamber

(BiOTRON; NK system, Osaka, Japan) under cycles of 14 h light at 23.5 ℃ and 10 h dark at 20.0 ℃. Plant above ground parts were weighed and after drying at 60 ℃ for 3 days, to obtain the fresh and dry weights, respectively. Effect of inoculation on plant growth was expressed as ratio (%) of the weights of inoculated plants against those of un-inoculated plants.

2.2.3. Statistical analyses

The statistical analysis was performed using IBM SPSS Statistics for Windows v.23.0 (IBM, Armonk, NY, USA). Data were subjected to the Student's *t*-test. A Tukey's honestly significant difference test with post-hoc comparison at the 5% confidence level was used to compare mean values among treatments. Experiments were performed with at least three replicates for each treatment.

2.3. Results

2.3.1. Bacterial growth under different temperatures and pH values

Figure 1 shows growth profiles of the six strains (*Rhizobium* sp. HRRK005, *Polaromonas* sp. HRRK103, *Variovorax* sp. HRRK170, *Mesorhizobium* sp. HRRK190, *Streptomyces* sp. HRTK192 and *Novosphingobium* sp. HRRK193) under different temperatures and pH ranges. All bacterial strains grew at 20 and 30 °C. HRRK170 showed the most active growth at both temperatures with a growth peak at 24 h after incubation. In addition, HRRK170 also showed the highest growth at 10 °C (Figure 1A). Only HRTK192 grew at 40 °C, but showed a lag time of 24 h at 20 °C. The growth of HRRK193 was active at 20 °C and close to that of HRRK170. However, its growth at 30 °C was considerably slower than that of HRRK170 and HRTK192. The other strains, HRRK005, 103, and 190, showed similar growth profiles and their growth was slower at both 20 and 30 °C, although HRRK103 grew slowly at 10 °C (Figure 1A). For pH, all strains grew at pH 6.0 to 9.0. Among them, HRRK170 exhibited active growth in a wide range of pH values (5.0 to 10.0) with a growth peak at approximately 24 h. HRTK192 also exhibited active growth from pH 5.0 to 9.0, which was close to that of HRRK170 at 24 h after incubation. However, growth was inhibited at pH 10.0. HRRK103 showed the highest growth at pH 6.0 and 10.0, but growth at other pH values was lower than that of HRRK170 at 24 h after incubation. Growth of HRRK005, 190, and 193 was slower than that of these three strains at pH 6.0 to 8.0, and HRRK005 showed no growth at pH 5.0 and 10.0. These results indicated that HRRK170 had the highest ability to grow in a wider range of temperatures (10 to 30 °C) and pH values (5.0 to 10.0) among the six strains.

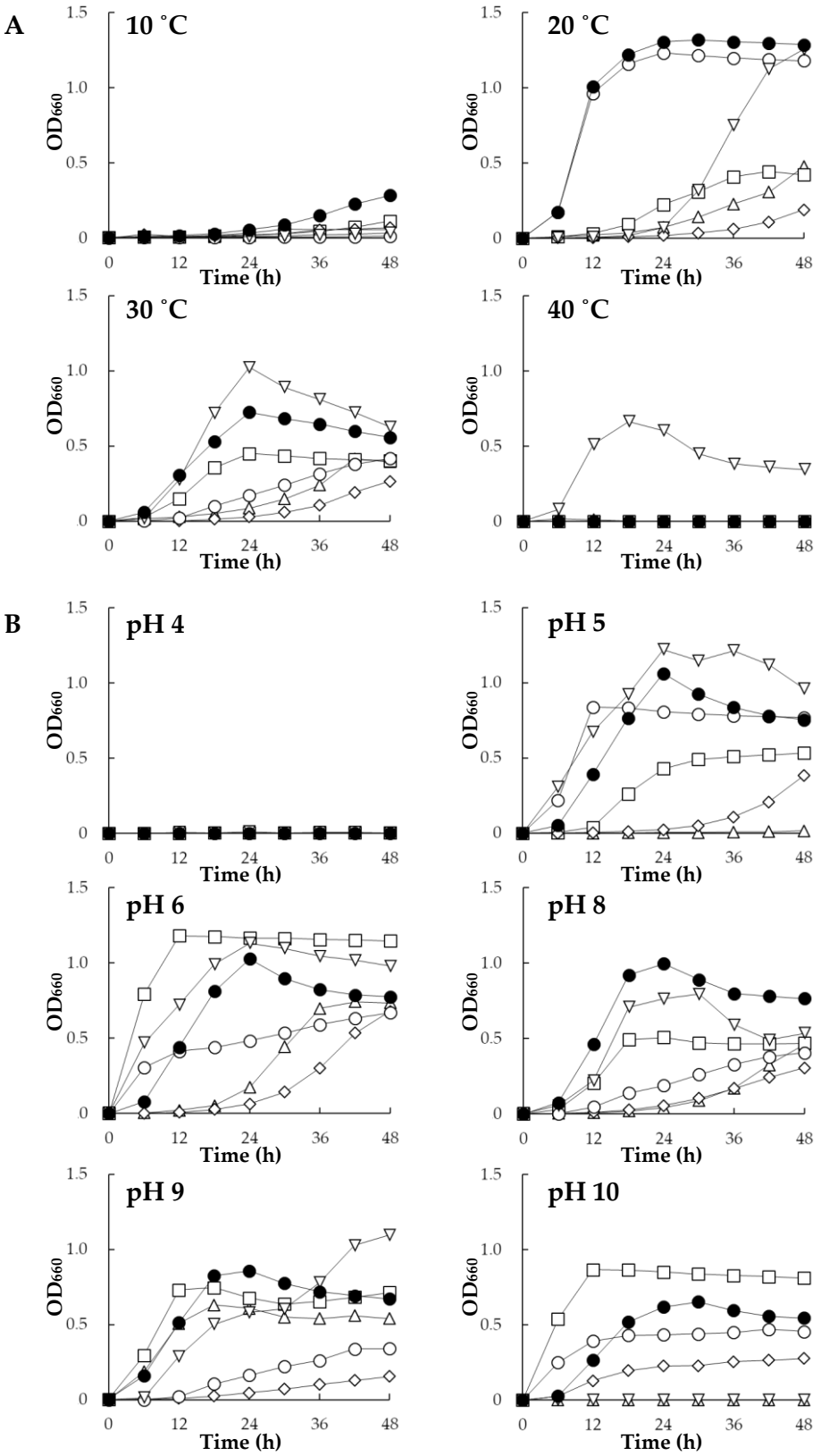

Figure 1. Growth of six plant growth-promoting bacteria under different temperatures (A) and pH values (B). Growth was monitored at OD_{660} using a biophotorecorder. Data are the means of three replicates. Symbols: ▵ , HRRK005; □, HRRK103; ●, HRRK170; ◊,RRK190; ▿ , HRTK192; ○, HRRK193.

2.3.2. Biochemical characteristics of PGPB strains

Based on the growth profiles of six strains under different temperatures and pH values as shown in Figure 1, biochemical activities of six strains were investigated at 30 °C and a neutral pH in order to evaluate their abilities to produce plant growth substances and enzymes (Table 1). All six strains produced siderophores, biofilm, and IAA. Particularly, HRRK170 exhibited higher production of these compounds and the production levels of siderophores and biofilm were the highest among the six strains (52.63 and 0.46, respectively) (Table 1). Regarding enzyme production, activity of ACC deaminase and β-1,3-glucanase was also observed in almost all of the six strains. However, the level of ACC deaminase produced by HRRK170 was significantly higher than that of the other strains ($p < 0.001$), and the level reached approximately 60.41 (nmol AKB mg^{-1} cell h^{-1}) (Table 1). Both cellulase and lipase activities were detected in HRRK005, HRRK170 and HRTK192, but chitinase activity was detected only in HRRK005 (Table 1). Results obtained from the biochemical analyses and growth profile different conditions indicated that HRRK170 had the highest potential as a plant growth promoter.

Table 1. Enzyme activities of six PGPB strains.

Strain	ACC deaminase (nmol AKB mg^{-1} cell h^{-1})	β-1,3-glucanase (U mL^{-1})	Cellulase (mm dia.)	Lipase (mm dia.)	Chitinase (mm dia.)
HRRK005	n.d.	0.02 ± 0.03^{a}	0.35 ± 0.15^{a}	1.34 ± 0.56^{b}	0.50 ± 0.08
HRRK103	0.98 ± 0.03^{a}	4.24 ± 0.10^{d}	n.d.	0.13 ± 0.42^{a}	n.d.
HRRK170	60.41 ± 2.47^{d}	0.62 ± 0.15^{b}	0.43 ± 0.25^{a}	3.24 ± 0.83^{c}	n.d.
HRRK190	5.86 ± 0.50^{b}	0.67 ± 0.30^{b}	n.d.	n.d.	n.d.
HRTK192	20.74 ± 1.92^{c}	0.46 ± 0.11^{b}	2.24 ± 0.40^{b}	3.36 ± 0.66^{c}	n.d.
HRRK193	19.58 ± 0.00^{c}	1.46 ± 0.11^{c}	n.d.	n.d.	n.d.

Values are means ± standard deviation. The significance of differences between means was compared using one-way analysis of variance. Mean values in the same column with common superscript letters are not significantly different ($p \leq 0.05$) by Tukey's honestly significant difference test. n.d.: not detected.

Table 2. Production of plant growth-promoting compounds of six PGPB strains.

Strain	Siderophore (%)	Biofilm (OD_{595})	IAA (μg mg^{-1} dry cell)
HRRK005	17.87 ± 0.23^{b}	0.26 ± 0.01^{a}	92.55 ± 0.55^{c}
HRRK103	13.14 ± 1.63^{a}	0.19 ± 0.02^{a}	74.31 ± 1.58^{b}
HRRK170	52.63 ± 1.70^{c}	0.46 ± 0.09^{b}	100.52 ± 2.02^{d}
HRRK190	13.07 ± 3.02^{a}	0.25 ± 0.03^{a}	92.54 ± 3.79^{c}
HRTK192	10.77 ± 1.45^{a}	0.43 ± 0.06^{b}	118.00 ± 0.82^{e}
HRRK193	11.99 ± 2.32^{a}	0.44 ± 0.09^{b}	16.43 ± 0.42^{a}

Values are means ± standard deviation. The significance of differences between means was compared using one-way analysis of variance. Mean values in the same column with common superscript letters are not significantly different ($p \leq 0.05$) by Tukey's honestly significant difference test.

2.3.3. Tissue localization of HRRK170 in sugar beet seedlings

Tissue localization of HRRK170 on root of sugar beet seedling was confirmed by GUS staining and viability test at 7 days post inoculation as visualized in Figure 2. GUS staining revealed that HRRK170 effectively localized on root surface and root hair. Bacterial viability test result showed that HRRK170 cells are physiologically active and localized on surface of root hair effectively. Similarly, SEM observation evidenced that cells localized on root surface area, probably HRRK170 was epiphytically associative to interact with plant in initial stage of interaction as showed in Figure 3.

2.3.4. Effect of HRRK170 on growth of sugar beet seedlings

In order to identify effect of HRRK170 different bacterial cell concentration on growth of sugar beet during the early interaction, seeds were inoculated with four different concentrations of cell in inoculant (Figure 4).

According to the result, when sugar beet seeds were inoculated with cell concentrations of 8.5×10^4 CFU mL^{-1}, 8.5×10^5 CFU mL^{-1}, 8.5×10^6 CFU mL^{-1} and 8.5×10^7 CFU mL^{-1} growth of sugar beet was improved growth by 119.5%, 119.0%, 128.0% and 138.7% in terms of fresh weight, and 124.2%, 110.0%, 119.8% and 116.8% in terms of dry weight, compared with the un-inoculated control, respectively.

Since the highest growth promotion as reflected in fresh weight increase ($p<0.05$) was recorded when 8.5×10^6 CFU mL-1 and 8.5×10^7 CFU mL^{-1} cell concentrated inoculants were applied, the higher cell concentration of inoculant was considered as suitable for further inoculation tests.

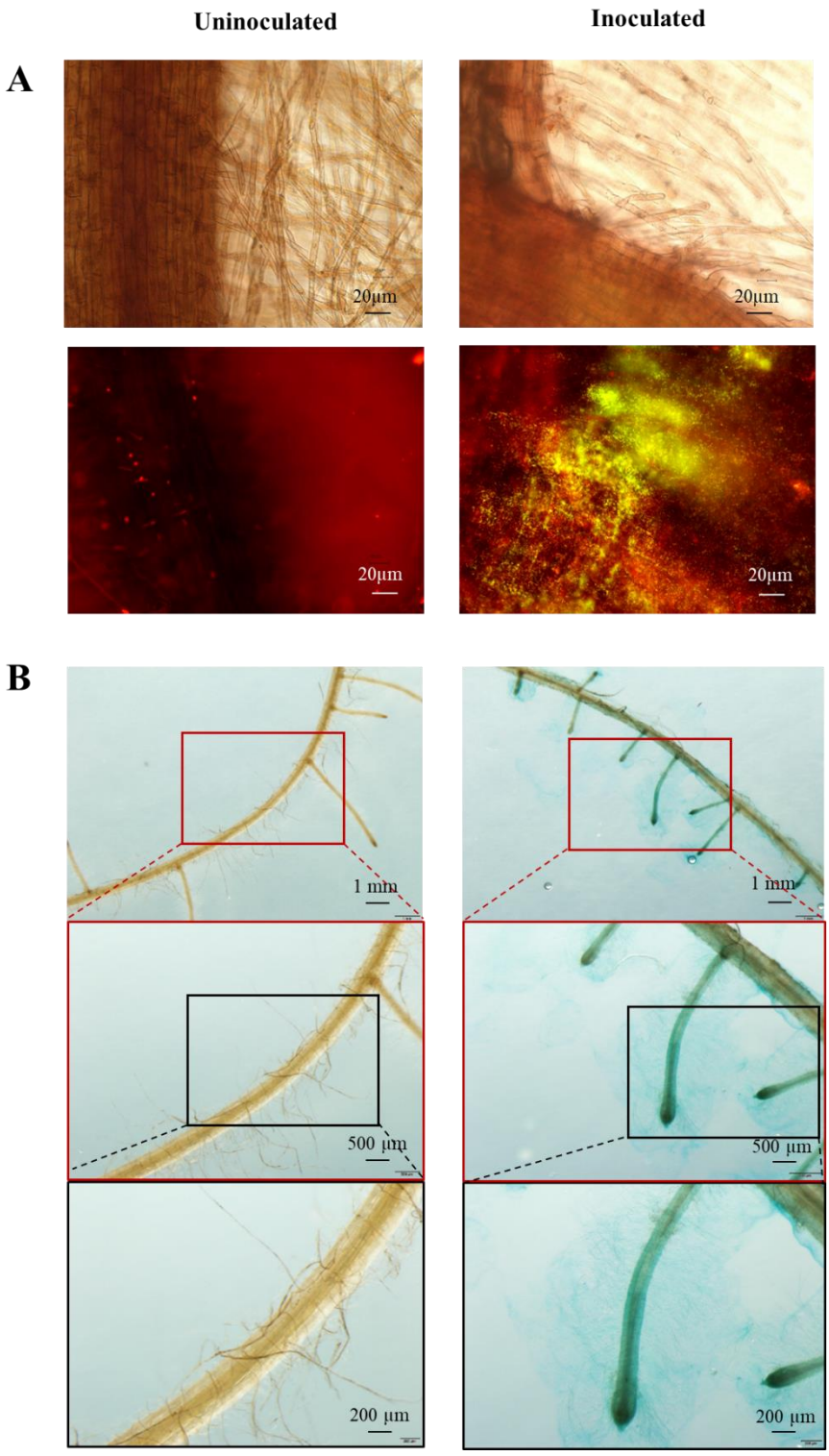

Figure 2. Tissue localization of *Variovorax* sp. HRRK170 cells in sugar beet seedlings. The localized cells stained by *Bac*Light viability kit (A) were observed under brighfield (upper side) and longpass filters (down side), using fluorescence microscope, and GUS-stained cells (B) were observed under stereomicroscope (right side) with comparison of un-inoculated plants (left side).

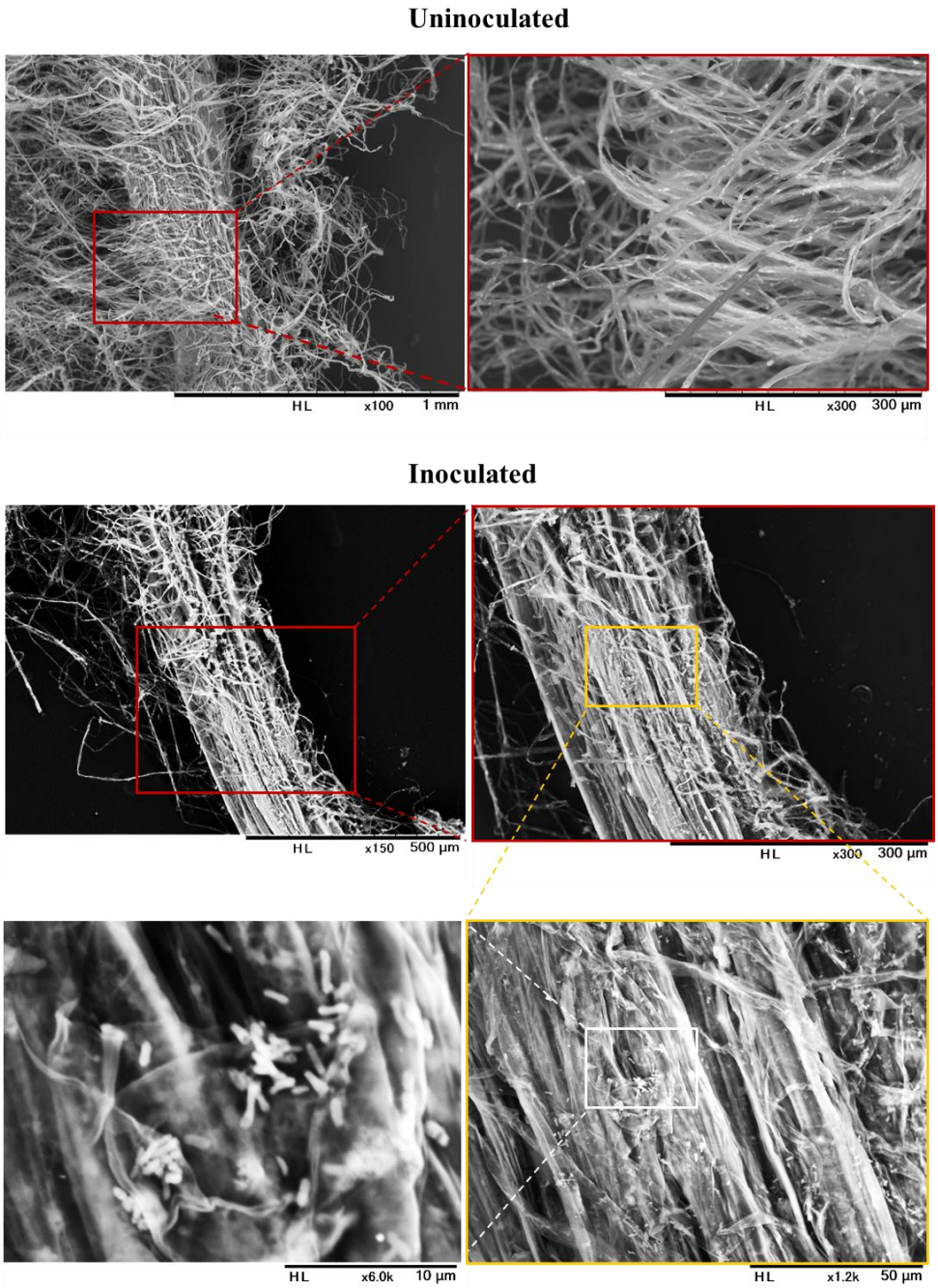

Figure 3. Scanning electron micrographs of sugar beet seedlings uninoculated or inoculated with *Variovorax* sp. HRRK170 cells. Enlarged boxes indicate area where bacterial cells localized densely.

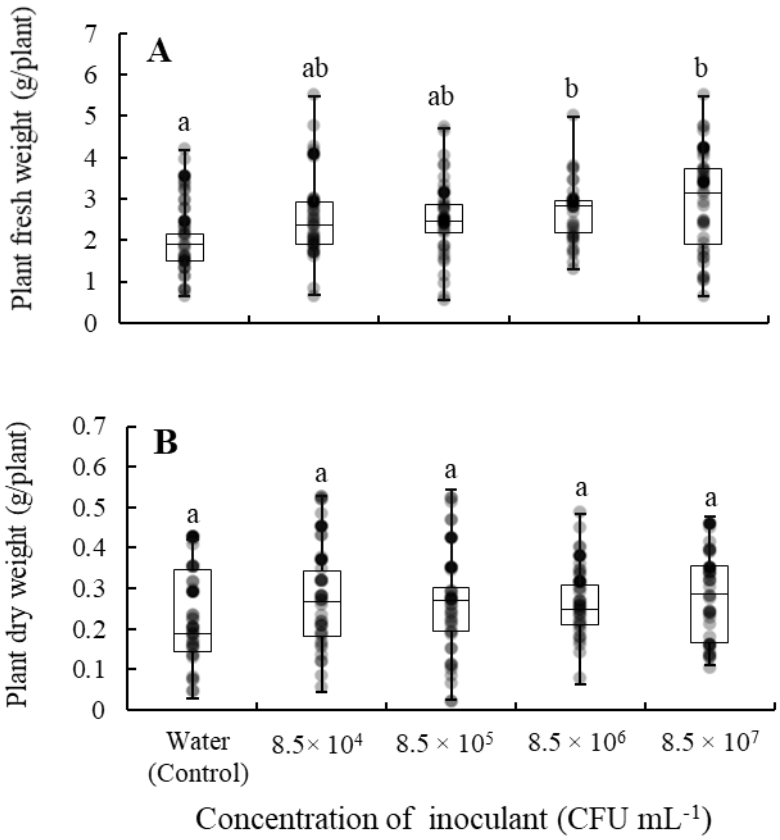

Figure 4. Effect of cell concentrations of *Variovorax* sp. HRRK170 on fresh weight (A) and dry weight (B) of sugar beet. Seedlings were inoculated with different concentrations of inoculant and grown for 4 weeks. Dry weight of plant was obtained by drying at 60ºC for 3 days. Horizontal bars show means ± standard deviations. Different letters above the bars indicate significant differences ($p \leq 0.05$; Tukey's test) between means. *P*-values determined by two-way ANOVA for concentration of inoculant.

2.4. Discussion

In this chapter of study, six potential plant growth-promoting bacterial strains, previously isolated from sugar beet, were screened as a bioinoculant. Siderophore production, ACC-deaminase and β-1,3-glucanase activities were detected in six strains by biochemical analyses and these characteristics were considered as prevail PGP traits observed in bacterial community isolated from sugar beet as demonstrated in the previous study (Tsurumaru *et al.*, 2015). In addition, biofilm and IAA production were identified in six strains, which are common PGP traits in beneficial rhizobacteria (de Souza, 2015).

Among all strains screened, HRRK170 had higher tolerance against lower temperature stress (10 ºC), while this condition inhibits growth of other strains. Also, HRRK170 showed the highest ability to produce plant growth substances and enzymes such as siderophores and ACC deaminase, respectively (Table 1). Zhou *et al.*, reported (2017) that *Variovorax paradoxus* isolated from sugar beet was able to promote growth of sugar beet growth due to its ACC-deaminase activity and abiotic stress tolerance.

The effect of HRRK170 strain on growth of sugar beet was evaluated using autoclaved pot soil (Figure 4) and inoculants with different cell concentrations. The highest growth promotion as reflected by increased fresh weight of plant was observed when inoculants with cell concentrations of 8.5×10^{6} CFU mL^{-1} and 8.5×10^{7} CFU mL^{-1} were applied. In practice, 1×10^{9} CFU mL^{-1} or higher than this concentration is common in liquid bacterial bioinoculant preparation (Bashan *et al.*, 2014; Schulz & Thelen, 2008). In laboratory condition, bacterial inoculant was commom to use bacterial suspension with cell concentration of 1×10^{8} CFU mL^{-1} (Vejan, Abdullah, Khadiran, Ismail, & Nasrulhaq Boyce, 2016). Therefore, we used bacterial suspension with the concentration of 8.5×10^{7} CFU mL^{-1} was as suitable concentration of inoculant for further inoculation tests in this study.

Furthermore, when plant tissue localization of HRRK170 on sugar beet seedlings was observed by *Bac*Light kit and GUS-staining, cells looked efficiently localized on rhizodermis. Cells could be observed directly by SEM at 7 days post-inoculation, suggesting this strain epiphytically associated with plant root during the initial interaction. Bacterial successful initial interaction with plant could be triggered by various factors including root exudates (Compant, Clément, & Sessitsch, 2010) plant cell wall component (Beauregard, Chai, Vlamakis, Losick, & Kolter, 2013), bacterial flagella driven chemotaxis, cell-density dependent quorum sensing and bacterial metabolites (Compant *et al.*, 2010; Hardoim, van Overbeek, & Elsas, 2008; Latour *et al.*, 2008). Among them, biofilm production can be crucial to infection processes (Compant *et al.*, 2005). For bacterial cell concentration of ininoculant, Larcher *et al.* (Larcher, Rapior, & Cleyet-Marel, 2008) reported that the initial dose of bacterial inoculant regulated the length of rapeseed root and shoot, suggesting that the cell number affects plant growth promotion. Suckstorff *et al.* (2003) also reported that *Stenotrophomonas* sp. showed plant growth-promoting behavior in a dose-dependent manner. On contrary, initial cell dose of inoculant also can be irrelevant to bacterial colonization value to plant (Juhnke, Mathre, & Sands, 1989).

Our study result suggested that initial cell concentration of HRRK170 may be less relevant to plant growth promotion as low cell concentration also showed tendency to improve plant growth. Probably, high biofilm production of HRRK170 led better attachment to plant tissue surface and enabled cells to efficiently localize on rhizodermis of seedlings and sustain beneficial interaction with plant.

Collectively, HRRK170 was the highest potential bioinoculant among six strains and its growth promoting effect could be contributed by multiple advantageous biochemical characteristics of PGPB and efficient plant tissue localization.

CHAPTER 3.

Plant growth promoting effect of *Variovorax* sp. HRRK170 on vegetables seedlings

3.1. Introduction

Enriching growing substrate with PGPB has a number of benefits for production of plants. Inoculation of PGPB can be effective way to reduce stress of seedlings and improve growth, especially after transplantation in production environment (G Teijeiro, Dodd, Elphinstone, Safronova, & Belimov, 2011).

Various studies have investigated the mechanisms of efficient plant–microbe interactions. Noirot-Gros *et al.* (2018) reported that the formation of bacterial biofilms, in which microbial cells live in self-synthesized extracellular polymeric substances, resulted in different formations, dispersion, and colonization patterns, followed by plant growth-promoting efficiency. This study results suggest that the effect of plant growth-promoting bacteria on plant growth is correlated with the number, dispersion, and colonization of infecting cells associated with their biofilm formations in plant tissues (Noirot-Gros *et al.,* 2018). Also some studies demonstrated that bacterial biochemical profile could alter interaction with plants. Also, the size of population on surface of root was also related with plant's growth response to the presence of bacteria (Dubeikovsky, Mordukhova, Kochetkov, Polikarpova, & Boronin, 1993).

Previous results in Chapter 2 suggested that HRRK170 was able to promote growth of sugar beet and it showed multiple beneficial characteristics as a PGPB inoculant. Therefore, in this chapter of study, HRRK170 was assessed as bioinoculant using various

vegetables seedlings for tissue localization and growth promoting profile. The results demonstrated that HRRK170 could function as plant growth promoter with efficient tissue localization capacity, but has an optimum cell density for its full function.

3.2. Materials and Methods

3.2.1. Morphological observation of HRRK170 cells

HRRK170 colonies grown on R2A agar plates were visualized on a scanning electron microscope according to the conventional method. Briefly, cells grown at 30 ºC for 48 h on R2A agar were smeared on a glass slide and, after immersion in 2% glutaraldehyde for 2 h, washed with 0.1 M phosphate buffer for 15 min, three times. Specimens were then treated with 50%, 75%, and 99.5% ethanol for 15 min, in this order, and immersed at 40 ◦C for 15 min three times in 99. 0% tertiary butyl alcohol. Then, samples were lyophilized, coated with gold using an MSP-mini magnetron sputter (VD, Ibaraki, Japan), and imaged on a Miniscope TM3030 (Hitachi Hi-Tech Co., Tokyo, Japan).

Swarming motility was examined by growing bacterial cells on semisolid (0.5%) nutrient agar (NA) plate. Bacterial suspension (OD_{600} = 0.1) was prepared from preculture and spotted (4 µL) on 0.5% NA plate. As a control, solid NA plate (1.5%) was used since bacterial cells only grow, but not swarm in that condition.

3.2.2. Inoculation test of HRRK170 and plant growth promotion

Plant growth promotion of HRRK170 on vegetable seedlings was evaluated as previously described in Chapter 2. PotAce N potting soil (Katakura & Co-op Agri, Tokyo, Japan) was used to assess the plant growth. Vegetable seeds [cabbage (*Brassica oleracea* L. cv. Harunami), lettuce (*Lactuca sativa* L. cv. Cisco), tomato (*Solanum lycopersicum* L. cv. Momotaro), radish (*Raphanus raphanistrum* L. cv. Taibyo sobutori), eggplant

(*Solanum melongena* L. cv. Senryo no.2), Chinese cabbage *(Brassica rapa* L. cv. Kigokoro 85, Kigokoro 65, Haregi 85, and Okiniiri), and green pepper (*Capsicum annuum* L. cv. Kyomidori, Kyonami, Ace, and Pitaro)] were purchased from Takii Seeds Co., Ltd. (Kyoto, Japan).

Seeds were sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite for 1 min, and then rinsed with sterilized distilled water (three times). Radish and eggplant seeds were treated ultrasonically (200 W; US-105, SND Co. Ltd., Japan) for 1 min (two times) before sterilization. Sugar beet seeds were left under running tap water for 24 h, sterilized with 70% ethanol for 1 min, 0.5% sodium hypochlorite containing 0.1% Tween 20 for 15 min, and then rinsed with sterilized distilled water for 15 min (three times). Sterilized seeds were sown on plug trays containing approximately 100 g soil and inoculated with 1 mL of 8.5×10^7 CFU mL^{-1} HRRK170 cells per 4 seeds. Seeds were covered with aluminum foil for 7 days, and then grown for three weeks in a growth chamber (BiOTRON; NK system, Osaka, Japan) under cycles of 14 h light at 23.5 °C and 10 h dark at 20.0 °C. Plant parts above ground were weighed before and after drying at 60 °C for 3 days, to obtain the fresh and dry weights, respectively. Effect of inoculation on plant growth was expressed as ratio (%) of the weights of inoculated plants against those of un-inoculated plants.

3.2.1. *Detection of tissue localization of HRRK170 in vegetables seedlings*

Observation of plant tissue localization of HRRK170 on seedlings was performed as abovementioned in Chapter 2. Sterilized seeds were sown on 1.5% agar plates were then transferred to 0.3% agar plates containing a 2000-fold dilution of HYPONeX 6-10-5 (HYPONeX, Osaka, Japan), and after inoculation with 100 µL of GUS-labeled HRRK170

cells (8.5×10^7 CFU mL^{-1}) per seedling, grown in a growth chamber (BiOTRON; NK system, Osaka, Japan) under the same conditions described above.

Afterwards, seedlings were GUS stained and microscopic observation of the localized HRRK170 cells in seedlings was conducted using a stereomicroscope (SZX16, Olympus Co., Tokyo, Japan). In parallel, agar (5%)-embedded sections of roots were sliced using a microslicer (DTK1000 ZERO1, Dosaka EM Co.,Ltd, Kyoto, Japan) and the localization of cells inside the plant tissues was observed under an inverted microscope (BZ-X700, Keyence Co., Osaka, Japan).

3.2.2. *Calculation of HRRK170 cell density using color development by GUS staining*

To quantify HRRK170 cell density by color development of GUS-staining solution, the correlation between HRRK170 cell density and the absorbance value (OD_{615}) of GUS-staining solution was examined. GUS-labeled HRRK170 cells were grown at 30 °C and 130 rpm, overnight in R2A broth containing the antibiotics described above, and serially diluted cell suspensions were prepared. After 2 mL of each diluted cell suspension was centrifuged at 4 °C and 18,000 x *g* for 5 min, the resultant cell pellets were suspended in 2 mL GUS-staining solution and stained as described above. After removing cells by centrifugation, the absorbance (OD_{615}) of the supernatant was measured using a spectrophotometer (Ultrospec 3100 pro, GE Healthcare Life Sciences, Buckinghamshire, UK).

In parallel, the cell number involved in each diluted cell suspension was also determined using the plate dilution method to make a correlogram with the OD_{615} values of GUS-staining solution. To calculate the plant-infected cell density of HRRK170 to plant, 10 seedlings inoculated with GUS-labeled HRRK170 cells were put into 20 mL of GUS-staining solution, and the absorbance (OD_{615}) of the solution was measured after

GUS staining. Then, the cell number was calculated using the correlogram, and cell density was expressed as the infected cell number per g fresh weight of plant.

3.2.3. Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics for Windows v.23.0 (IBM, Armonk, NY, USA). Data were subjected to the Student's *t*-test. A Tukey's honestly significant difference test with post-hoc comparison at the 5% confidence level was used to compare mean values among treatments. Experiments were performed with at least three replicates for each treatment.

3.3. Results

3.3.1. Morphological characteristics of HRRK170 cells

Figure 5 shows morphological characteristics of *Variovorax* sp. HRRK170 grown on an R2A agar plate. HRRK170 cells grown on R2A agar were gelatinous, glistening, yellowish, and robustly proliferative. However, the colony shape was obscure and smeary (Figure 5A).

In addition, cells were straight or slightly curved rods, approximately 1.5 μm long, and embedded in a mucilaginous layer due to its ability to produce biofilm, as visualized using scanning electron microscopy (Figure 5B). Swarming motility of HRRK170 was assayed on NA plate (Figure 6). Cells grown on 1.5% NA induced growth with 0.53 cm (Figure 6A), while cells on 0.5% NA plate grew and expanded to 1.33 cm swarming zone (Figure 6B). Biofilm production and swarming activity may provide HRRK170 cells with better attachment and competitiveness in soil condition.

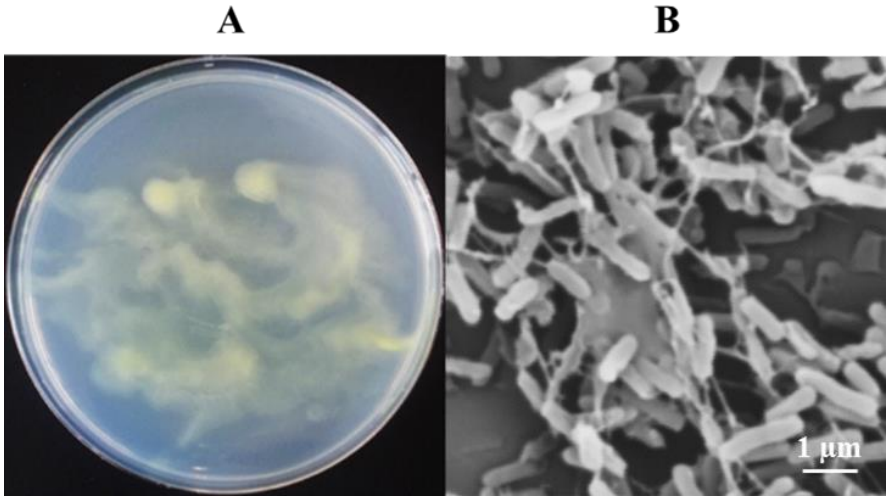

Figure 5. Morphological characteristics of *Variovorax* sp. HRRK170 cells. (A) Cells grown on R2A agar at 30 °C for 48 h. (B) Microscopic observation cells grown on R2A agar using a scanning electron microscope.

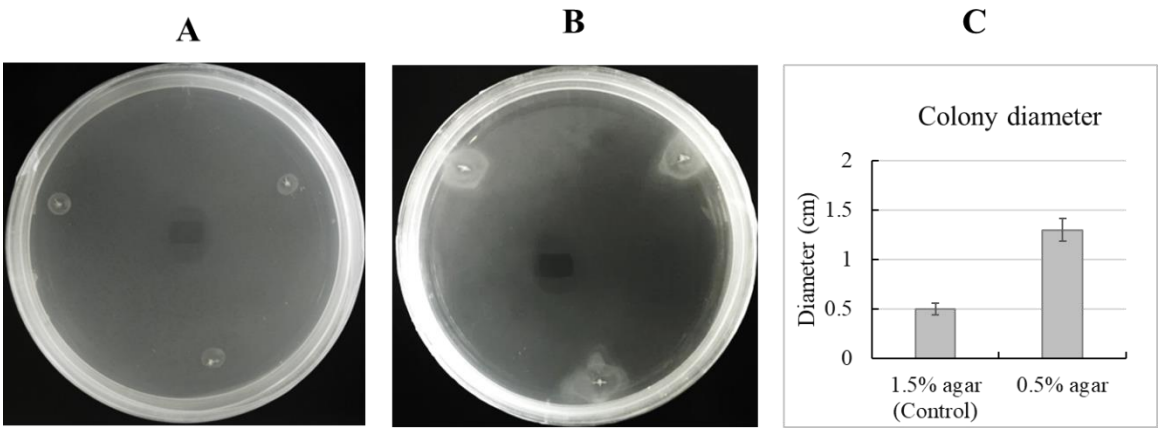

Figure 6. Swarming activity of *Variovorax* sp. HRRK170 cells
(A) grown on NA agar (1.5%) as a control at 30 °C for 24 h (control).
(B) grown on NA agar (0.5%) at 30 °C for 24 h.
(C) Colony diameter (cm) was measured in triplicate, reported as mean ±SD

3.3.2. Effect of HRRK170 on the growth of vegetable seedlings

Figure 7 shows the inoculation effect of *Variovorax* sp. HRRK170 on the growth of seven vegetable seedlings in addition to sugar beet, which was used as a reference. The results showed that HRRK170 significantly promoted the growth of Chinese cabbage and green pepper as well as sugar beet compared with the uninoculated control (ratio (%) of the weights of inoculated plants against those of uninoculated plants: Chinese cabbage, 142.4 and 157.1; green pepper, 149.0 and 200.2; sugar beet, 124.1 and 137.8, in terms of fresh and dry weights, respectively). The growth of eggplant also significantly increased with the inoculation of HRRK170 in terms of dry weights (ratio (%) against the uninoculated control: 118.9). In contrast, the growth of tomato and lettuce significantly decreased in terms of dry weights, particularly that of tomato, which was significantly decreased with the inoculation in terms of both fresh and dry weights (ratio (%) against the uninoculated control: 72.5 and 53.0, in fresh and dry weights, respectively).

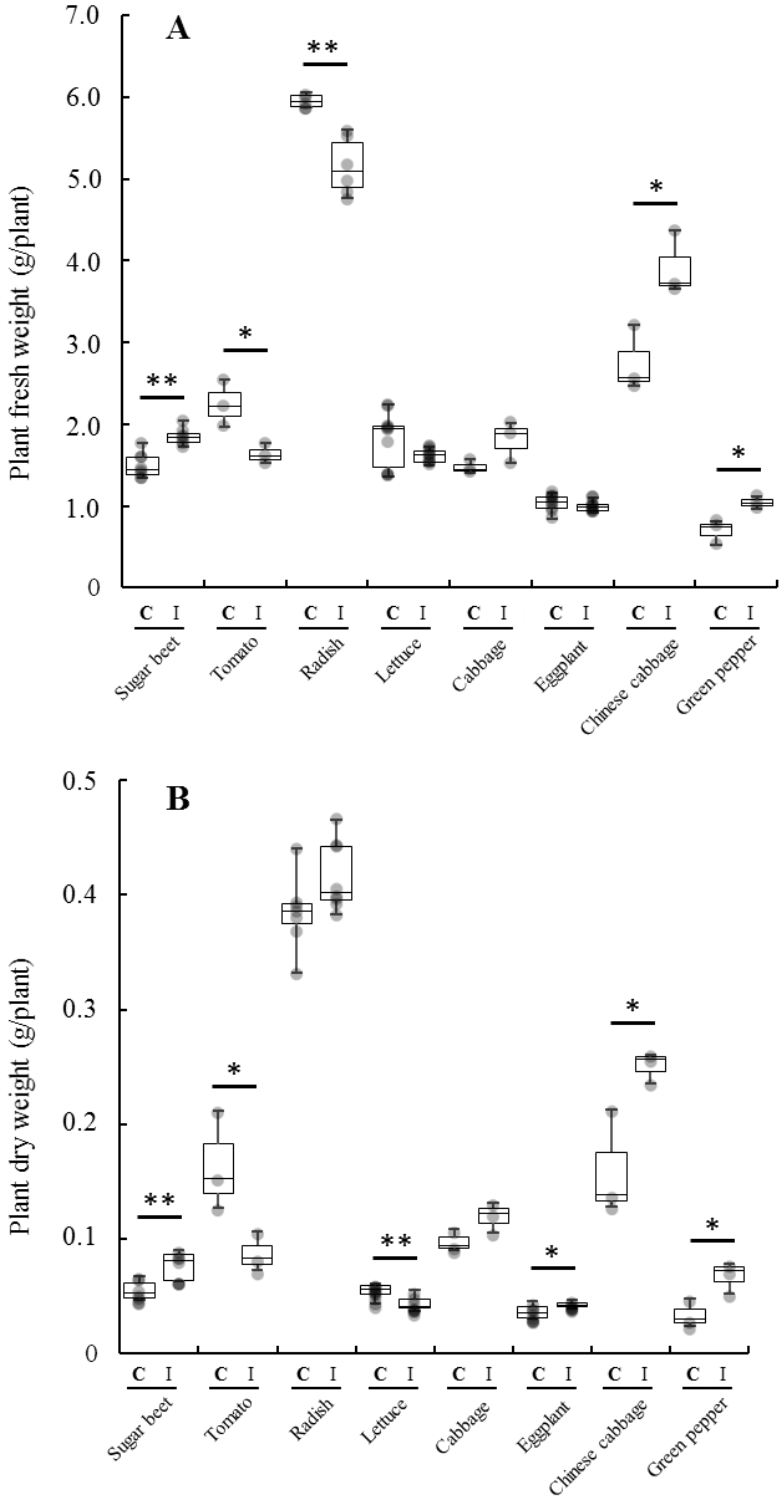

Figure 7. Effect of *Variovorax* sp. HRRK170 on the growth of vegetable seedlings after 4 weeks post inoculation. Plant fresh weights (A) and dry weights (B) are shown as means ± standard deviation. Mean values were compared by t-test. Asterisks indicate significant (*, $p < 0.05$; **, $p < 0.01$) differences between inoculated (I) and uninoculated control (C).
Host plants used: tomato cv. Momotaro, radish cv. Taibyo sobutori, lettuce cv. Cisco, cabbage cv. Harunami, eggplant cv. Senryo 2 go, Chinese cabbage cv. Haregi 85, green pepper cv. Kyonami, and sugar beet cv. Rycka as a reference.

3.3.3. Plant tissue localization of HRRK170 in vegetables seedlings

Figure 8 shows localization of HRRK170 in plant tissue during its initial interaction with the seven vegetable seedlings and the sugar beet as a reference, 7 days post-inoculation. HRRK170 colonized all plants by 7 days post-inoculation, but was localized only in plant roots as shown in GUS-stained plant tissues. In addition, the localization profiles of HRRK170 in roots were divided into two types: entire and partial. The colonization throughout the roots was observed in Chinese cabbage (a), cabbage (b), lettuce (c), and radish (d), whereas partial but prominent colonization in the roots was in green pepper (spot-like colonization) (e), eggplant (uneven colonization) (f), tomato (uneven colonization) (g), and sugar beet (colonization including root hairs) (h) (Figure 8). Next, the transverse root sections were prepared and internal infection of HRRK170 into the plant tissue was assessed using GUS staining. Results indicated that HRRK170 localized to the epidermis and root hairs at 7 days post-inoculation (Figure 8).

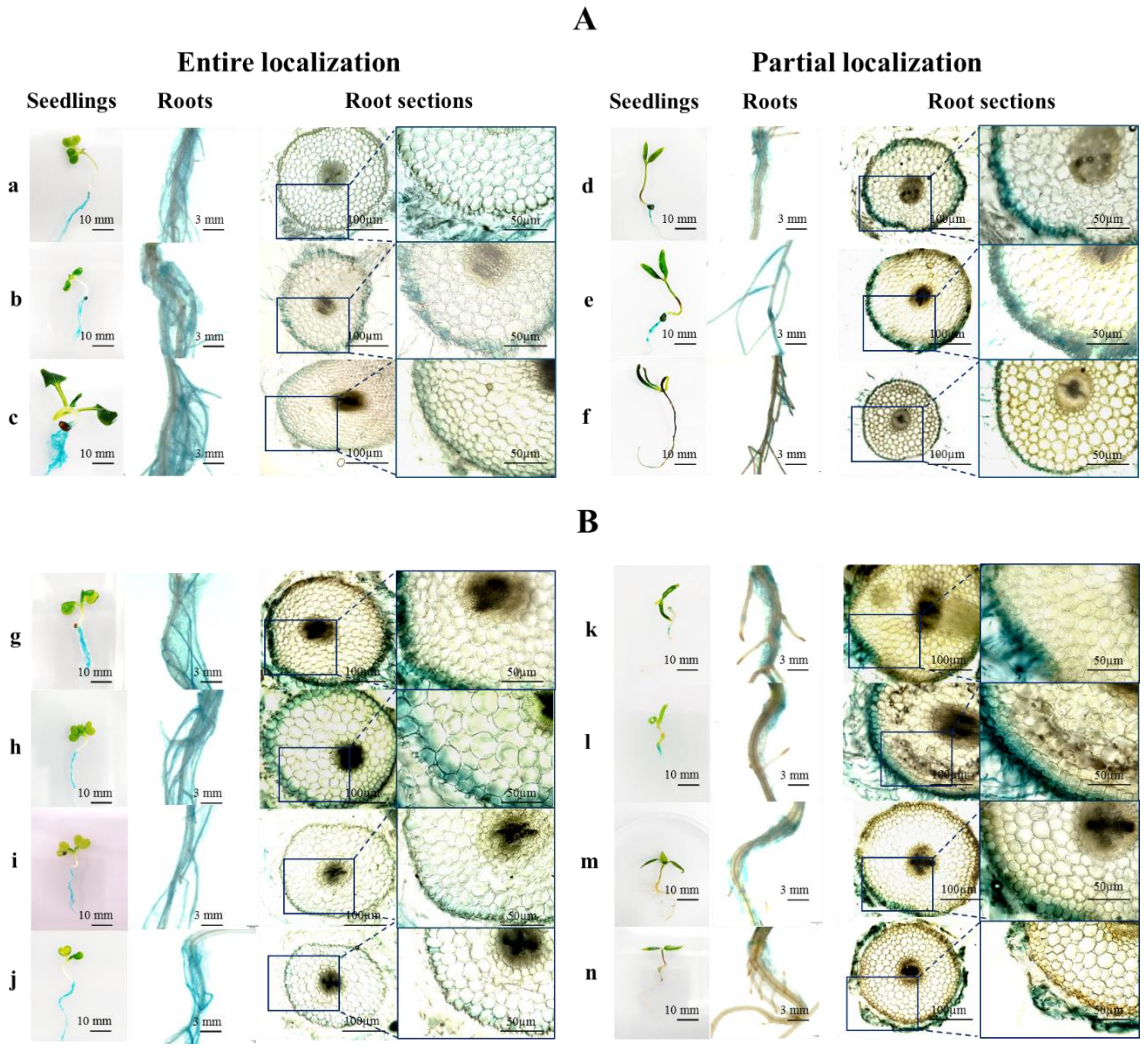

Figure 8. Tissue localization of *Variovorax* sp. HRRK170 in five vegetable seedlings and sugar beet as a reference (A) and for the four cultivars each of Chinese cabbage and green pepper (B). The cells localized in the seedlings at 7 days post-inoculation were detected using GUS staining, and entire (left panels) and partial (right panels) localization was observed using a stereomicroscope.
A: Tissue localization on (a) cabbage (cv. Harunami), (b) lettuce (cv. Cisco), (c) radish (cv. Taibyo sobutori), (d), eggplant (cv. Senryo 2 go), (e) tomato (cv. Momotaro), and (f) sugar beet (cv. Rycka) as a reference.
B: Tissue localization on Chinese cabbage (left panel) for (j) cv. Haregi 85, (h) cv. Okiniri, (j) cv. Kigokoro 85, (i), Kigokoro 65 and Green pepper (right panel) for (k) Green pepper (cv. Kyonami), (l) cv. Pitaro, (m) cv. Kyomidori, (n) cv. Ace.
Boxes show the enlarged regions in insets. Photos of seedlings, roots, and root sections were obtained using a digital camera, stereomicroscope, and inverted microscope, respectively.

3.3.4. *Evaluation of HRRK170 cell density localized on the plant roots*

Since HRRK170 promotes or represses plant growth, as shown in Figure 7, we tested the possibility that the cell density localized in the plant roots may explain this contradictory effect. Figure 9A shows the color development of GUS-staining solution after staining of GUS-labeled HRRK170 cells localized in the roots. This finding prompted us to estimate the cell density using the staining intensity because this method is faster and more convenient than the agar plate dilution method, which is generally used to count cell number.

To subject the staining intensity to colorimetry, the most appropriate wavelength in absorption spectra was first obtained from the color-developed solution caused by GUS-stained HRRK170 cells, i.e., absorbance at 615 nm was used as an index of staining intensity (Figure 9B). Then, the correlation between the cell density of HRRK170 (cell number per mL) and OD_{615} was assessed (Figure 10). Interestingly, the result indicated that OD_{615} was linearly correlated with the cell density obtained from plate dilution method, although absorbance data below or above 0.128 were fitted to different correlation coefficients in order to achieve better linearity (Figure 10). The cell densities were then estimated using OD_{615} with this correlation curve.

Figure 11A shows the relationship between cell density of HRRK170 (cell number per g of plant) and the plant growth ratio (percent of the inoculated plant weights against uninoculated control, which was calculated from the plant weights obtained in Figure 7) for seven vegetables with sugar beet as a reference. Results indicated that the plant growth ratio (%) was closely related to the cell density and had its optimum values for the plant growth promotion, i.e., green pepper and Chinese cabbage exhibited higher values of both growth ratio and cell density than radish and lettuce, and their growth ratios were significantly higher than those of the other five vegetables (Figures 7 and 11A).

Interestingly, the cell densities of cabbage, eggplant, and tomato were higher, while their growth ratios were lower than those of green pepper and Chinese cabbage. Notably, the growth of tomato ratio was considerably reduced, although it was infected with the highest number of cells.

Next, the relationship between the plant growth rate and cell density was sought whether it would also be present in several cultivars of the same host plant. Therefore, four cultivars of Chinese cabbage and four of green pepper were examined if infected cell density was related with its growth response. Interestingly, a similar relationship was found in these cultivars, i.e., the plant growth ratio reflected a cell density-dependent manner with its optimum values (Figure 11B). Particularly, for green pepper, the growth was increased with an increase in the cell density for the three cultivars (Ace, Kyomidori, and Kyonami, in this order), but decreased for the cultivar Pitaro, for which the cell density was the highest among the four cultivars (Figure 11B). These results indicated that HRRK170 promoted plant growth within a certain range of cell densities, i.e., at an optimum cell density for its full function in the early interaction with host plants.

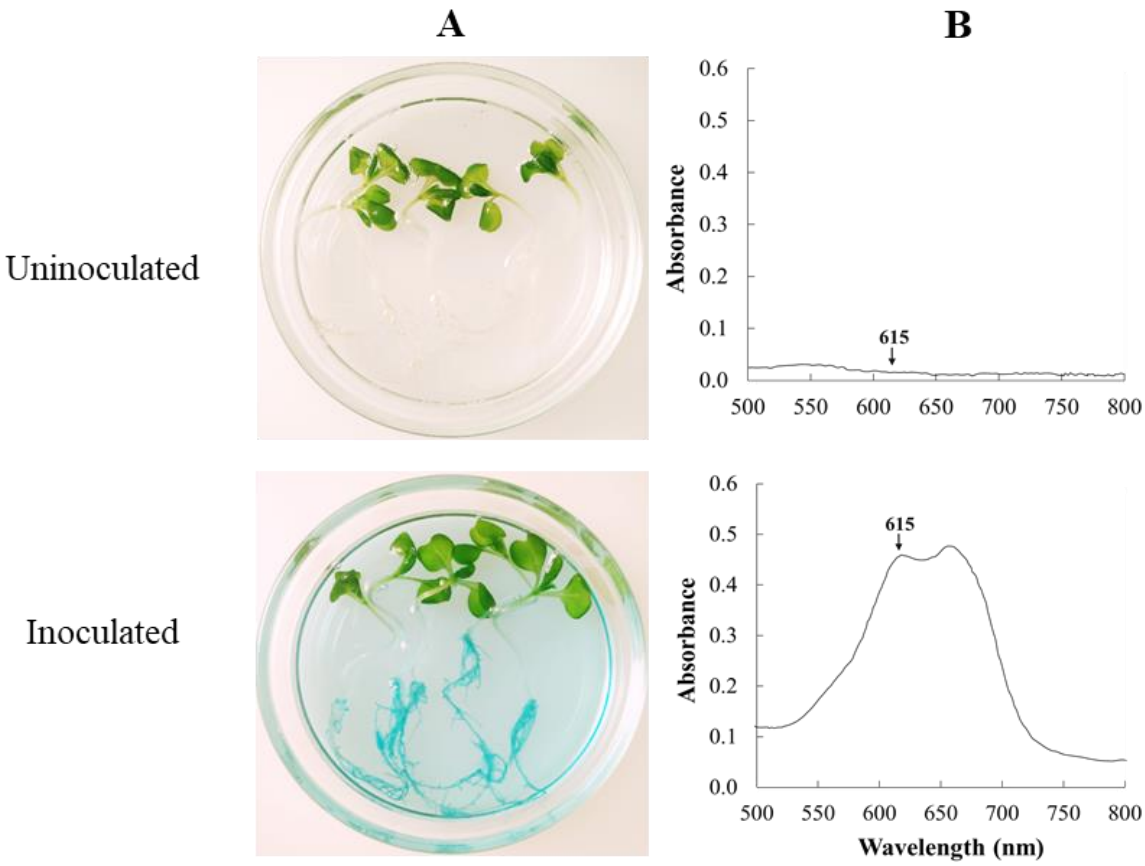

Figure 9. Color development in the reaction solution of GUS-stained *Variovorax* sp. HRRK170 localized in the roots (Chinese cabbage, cv. Haregi 85) (A) and an absorption wavelength of the color-developed reaction solution (B). The absorbance of indigo-blue chromophore produced by the enzymatic hydrolysis of β-glucuronidase (OD_{615}) is shown by the arrows.

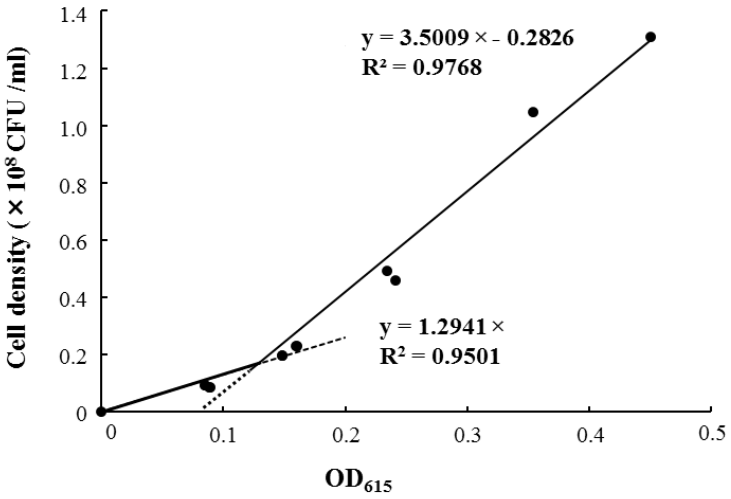

Figure 10. Correlation between *Variovorax* sp. HRRK170 cell density and absorbance value (OD_{615}) of the reaction solution colored using GUS staining.

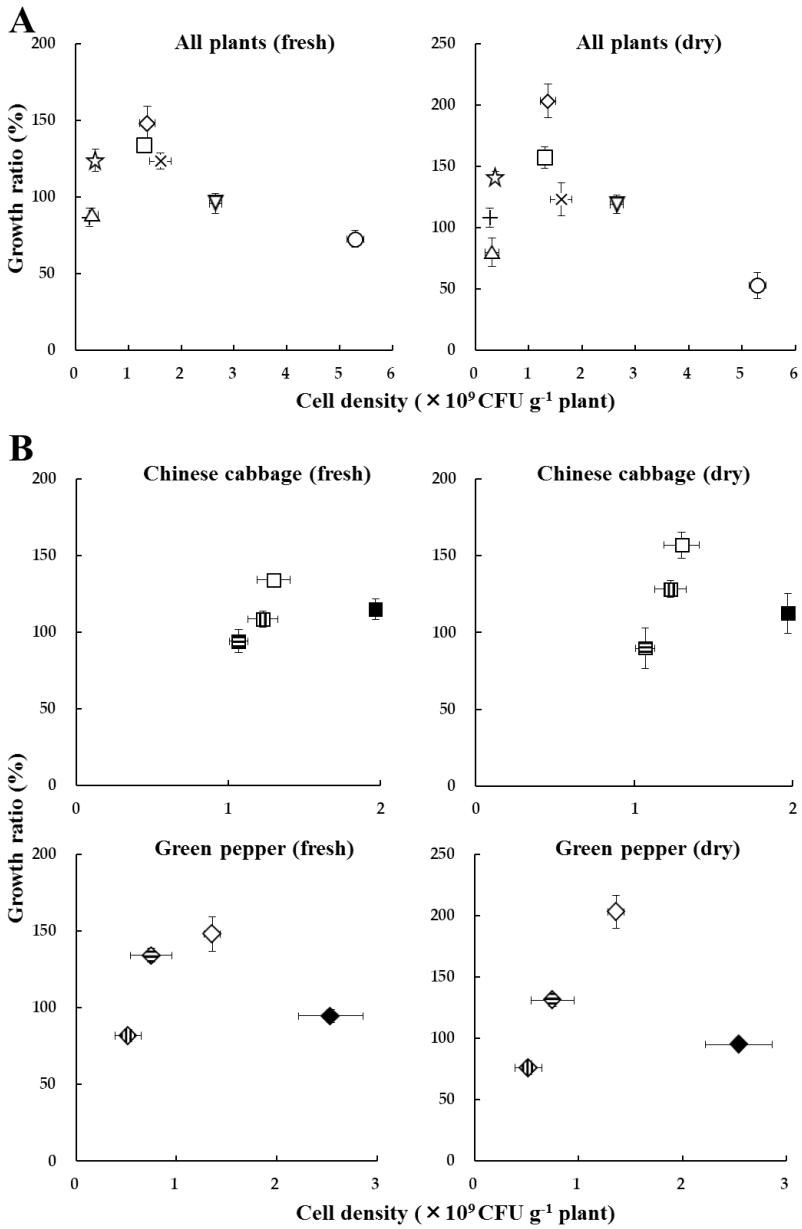

Figure 11. Relationship between *Variovorax* sp. HRRK170 cell density and growth ratio (%) of seedlings for seven vegetables, with sugar beet as a reference (A) and for the four cultivars of both Chinese cabbage and green pepper (B). Vertical axes show the ratio (%) of the plant weight inoculated with *Variovorax* sp. HRRK170 to that without it (growth ratio for fresh and dry weights is shown on the left and right, respectively). Cell density was calculated as the cell number per g of plant using the correlation curve described in Figure 10. Vertical and horizontal bars show means ± standard deviation. Symbols used
(A): ○, tomato cv. Momotaro; +, radish cv. Taibyo sobutori; △, lettuce cv. Cisco; ×, cabbage cv. Harunami; ▽, eggplant cv. Senryo 2 go; □, Chinese cabbage cv. Haregi 85; ◇, green pepper cv. Kyonami; ☆, sugar beet cv. Rycka as a reference. (B): Chinese cabbage: □, cv. Haregi 85; ▤, cv. Kigokoro 65; ▥, cv. Kigokoro 85; ■, cv. Okiniiri. Green pepper: ◇, cv. Kyonami; ◈, cv. Kyomidori; ◈, cv. Ace; ◆, cv. Pitaro.

3.4. Discussion

The type species, *V. paradoxus*, is known to have straight to slightly curved rods, and the colonies on nutrient agar are normally convex, glistening, shiny, and yellow or greenish yellow in color. Additionally, growth factors are not required because of the availability of a wide variety of organic compounds (Willems, Mergaert, & Swings, 2015). Previously, genomic analyses by Han *et al.* (2011) revealed that *V. paradoxus* S110 was metabolically diverse and highly adaptable to various environmental conditions, and thus may support plant growth and degrade toxic compounds (Han *et al.*, 2011). Belimov *et al.* (2009) reported that positive effect of *V. paradoxus* 5C-2 on growth of pea in both well watered and drying soil. In this chapter, plant growth promoting capacity of HRRK170 was assessed using vegetables seedlings.

HRRK170 colonized on the roots of all seven vegetable seedlings and sugar beet at 7 days post inoculation, significantly promoted the growth of two vegetable seedlings (Chinese cabbage and green pepper) as well as the sugar beet, and also showed upward an tendency for two vegetable seedlings (cabbage and eggplant) (Figures 7 and 8). However, HRRK170 failed to function as a plant growth promoter for the other three vegetables (tomato, radish, and lettuce) (Figure 7). The colonization profiles of HRRK170 showed two different types (entire and partial) but did not seem to be correlated with the inoculation effects of HRRK170 (Figures 7 and 8). To elucidate the contradictory behavior of HRRK170 the number of HRRK170 cells infecting each plant was estimated in order to evaluate the relationship with plant growth promotion. Typically, cell numbers of the infecting strain are calculated via the plate dilution method after crushing plant tissues. However, since GUS-stained HRRK170 cells were found to cause color development due to GUS-staining solution, the cell number was estimated by the absorbance value (OD_{615}) because this method provided more accurate information

after only 3.5 h incubation without crushing the plant tissues. The substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), for β-glucuronidase produces an insoluble, intense, indigo-blue chromophore (OD_{615}) after enzymatic hydrolysis (Sigmaaldrich, 2019). Interestingly, the color development of the reaction solution is based on the production of this indigo-blue chromophore caused by enzymatic hydrolysis of X-Gluc, and its absorbance value (OD_{615}), which is less than approximately 0.45, correlated with the cell number, indicating that it could be estimated using this absorbance value (Figure 10). However, it remains unclear why this correlation curve does not show a simple linearity, since it is considered that the *gusA* gene encoding ß-glucuronidase is constitutively expressed from the promoter (Wilson *et al.*, 1995). It may also be possible that the *gusA* expression was affected by cell number in such a way as to be lowered under higher cell number.

The cell density of HRRK170 localized in the plant roots (cell number per g of plant) could be estimated using this correlation curve because most of the cells localized on the epidermis at 7 days post-inoculation (Figure 8). This study showed that HRRK170 promoted plant growth within a certain range of cell densities, indicating that it has an optimum cell density for its full function. Excess cell density may cause to lose symbiotic balance with the host plant, whereas low affinity with the host plant results in low density of infecting cells. Besides, prompt counting of cell number based on GUS-staining methodology would simplify the evaluation of infecting cell density in plants and provide new insight into plant–microbe interactions.

Our results indicate that infecting cell density is an important factor in determining whether or not the bacterial strain fully functions to promote plant growth, and optimum cell density must be considered for the application of plant growth-promoting

bioinoculants to host plants. Further studies are needed to clarify how the infecting cell density affects plant growth promotion.

CHAPTER 4.

Mitigation of salt and drought stresses in Chinese cabbage by *Variovorax* sp. HRRK170

4.1. Introduction

Resilient ecosystem and food security are the most concerning agricultural issue worldwide (Raza *et al.*, 2019). Plants are naturally exposed to numerous environmental stresses that affect growth, resulting in massive losses in production (Rejeb, Pastor, & Mauch-Mani, 2014). Temperature, drought, soil pH and salt stressors are major abiotic stressors for plant. In particular, drought and salt stresses result in morphological, biochemical and molecular modification in plants (Sahin *et al.*, 2018) and greatly impact agricultural productivity worldwide (Forni *et al.*, 2017). Therefore, climate-smart and tolerant crops are being developed globally.

Although the external application of chemicals such as caffeic acid, sodium polyacrylate, jasmonic acid, genistein, chitosan, humic acid, glycine betaine, proline and nutrients, as well as osmo-protectants have been used to improve plant resilience, the optimal yield sustainability has not being achieved through the use of chemicals alone in practice (Enebe & Babalola, 2018). Thus, mitigating and alleviating stress impact via integrated application of microbes and other stress mitigating factors may be the most effective strategy to boost yield and protect it from climate stresses (Figure 12).

When plant experiences stress, such as salinity, heating, chilling, wounding, nutritional stress, and pathogen infection, ethylene level increases markedly. Although some plants require ethylene synthesis for some developmental processes, (Glick, 2005)

maintaining a high level ethylene accumulation ultimately induces physiological disorder and inhibits growth and development (Nadeem, Zahir, Naveed, & Ashraf, 2010). Therefore, ethylene levels may be an indicator of stress in plants (Belimov *et al.*, 2009). Some plant-associated microbes produce ACC-deaminase, which reduces ethylene levels in tissue by cleaving the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) into α-ketobutyrate, resulting in elevated resilience to abiotic stresses in plants. Bacteria can employ several strategies not only producing ACC-deaminase, but also increasing osmolytes (*e.g.* proline) accumulation, stomatal conductance, maintaining higher photosynthetic capacity and balancing ionic homeostasis of host plants. Hence, microbes can play important role in facilitating plant growth under climate-induced stress (Penrose & Glick, 2003).

Additionally, bacterial IAA production, a common auxin hormone (more than 80% of bacteria in rhizosphere are able to produce IAA) (Sarkar & Laha, 2013), is directly involved in many aspects of plant physiology. For example, IAA stimulates root elongation, which enhance nutrient uptake and improves of overall growth compared with un-inoculated plants under stressed condition (Van Puyvelde *et al.,* 2011). When undergoing salt stress, several cellular changes occur in plants, such as ion homeostasis, ionic toxicity, membrane alterations, and production of free radicals, which limits growth. Exopolysaccharides and siderophores produced by plant-associated microbes help to prevent movement of toxic ions, regulate water transport, and ionic balance, and control pathogens population under stressed condition (Radhakrishnan, Hashem, & Abd Allah, 2017). In soils subjected to drought, plants become more dependent on microbial activity, which is able to increase nutrients and water uptake (Marulanda, Barea, & Azcón, 2009).

Chinese cabbage is susceptible to salt stress (glycophyte) (Qiu *et al.,* 2017) and least tolerant to drought stress among brassica plants (Pavlović *et al.,* 2018) owing to the

rapid growth and production of large amount of biomass (J. Wang *et al.*, 2019). It is common and widely consumed vegetable; however, its growth is severely inhibited under conditions of drought (Lee *et al.*, 2018) and salt (Qiu *et al.*, 2017). The salt tolerance mechanism of Chinese cabbage remains unclear (Qiu *et al.*, 2017) because of its complexity. In addition, when Chinese cabbage was exposed to salt stress, several genes such as those encoding auxin-responsive proteins are downregulated, suggesting that the reduced plant size and root length of Chinese cabbage can be partially caused by limited auxin productions (Qiu *et al.*, 2017). Regarding drought stress, previous studies have demonstrated that internal IAA level of Chinese cabbage was significantly reduced when the plant is stressed (Pavlović *et al.*, 2018). Therefore, IAA production of the HRRK170 was examined as it may play an advantageous role by stimulating plant growth under salt and drought stressed condition.

The results in previous chapter suggested that HRRK170 had the most potential as a bioinoculant among bacterial strains isolated from sugar beet and functioned as a growth promoter to Chinese cabbage and green pepper seedlings. Thus, the study described in this chapter aimed to investigate the interaction of HRRK170 with two cultivars of Chinese cabbage (Kigokoro 85 and Haregi 85) by challenging under polyethylene glycol (PEG) induced drought and NaCl induced salt stresses. The result revealed that the HRRK170 can play an important role in mitigating salt and drought stresses through increased amount of plant growth promoting compounds, while cells stably localized on plant root surface area. Although the long term effect of this strain was not investigated, it was found that HRRK170 can act as plant growth promoter and protect from damage caused by alt and osmitic stresses in early growth stage of seedlings under stressed conditions.

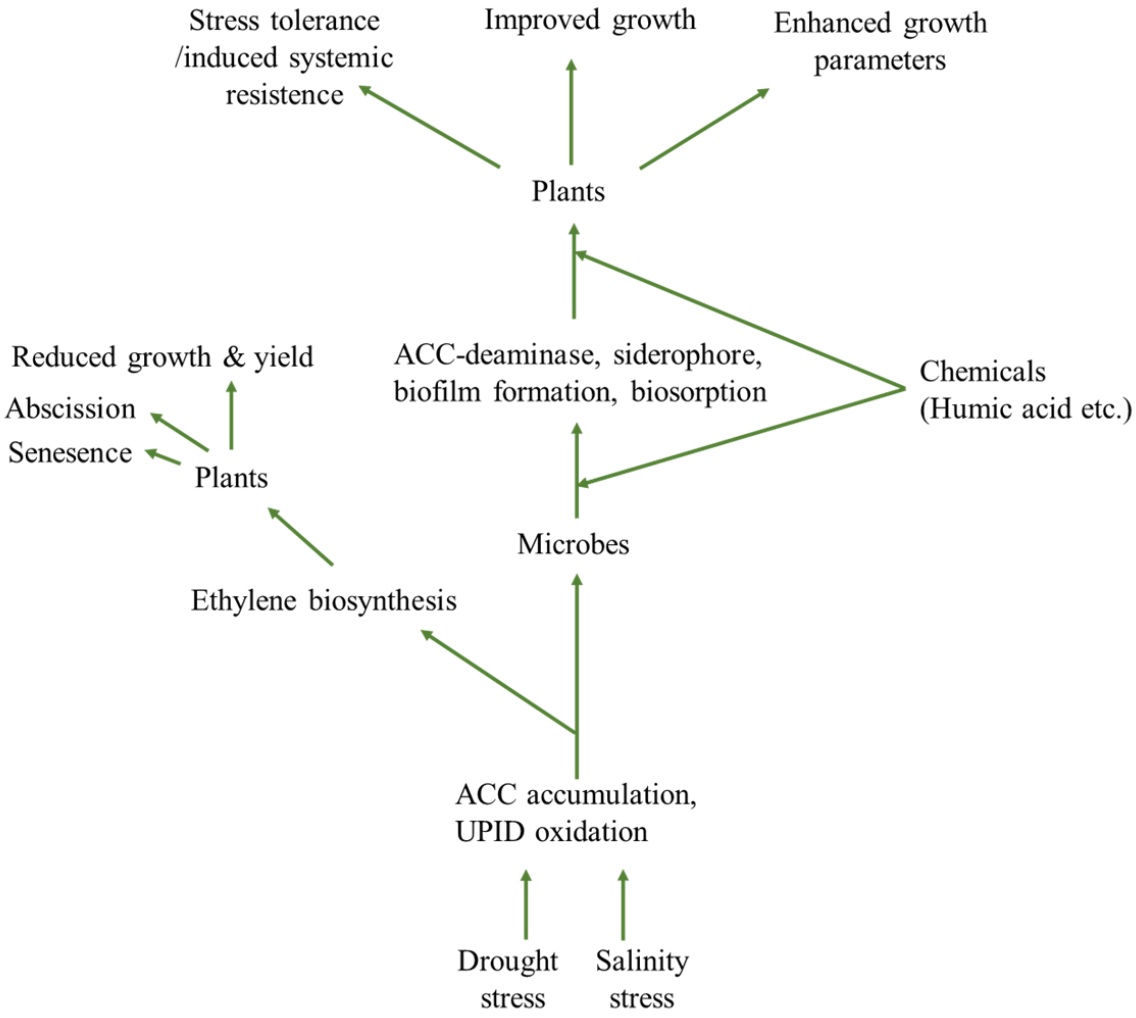

Figure 12. Scheme of the interactions of microbes, useful chemicals, and their overall influence in plant stress tolerance, growth, and productivity **(Forni *et al.*, 2017)**.

4.2. Material and methods

4.2.1. Bacterial growth condition and salt and drought tolerance

Wild-type HRRK170 and GUS-labeled HRRK170 cells were grown and maintained in R2A broth (BD, Sparks, MD, USA) as described in Chapter 2. To examine bacterial tolerance to stressors, medium was prepared by adding 0.5%, 1% and 1.5% NaCl (FUJIFILM Wako Chemicals, Japan) or 10% and 20% polyethylene glycol (PEG) – 6000 (FUJIFILM Wako Chemicals, Japan) (-0.11 MPa and – 0.33 MPa) to R2A broth medium. Bacterial cells were transferred from overnight grown preculture to medium and grown at 30ºC. Growth was monitored at 660 nm using a biophotorecorder (TVS062CA; Advantec Co., Tokyo, Japan).

4.2.2. Biochemical characteristics of HRRK170 under stresses

IAA production was estimated as described in Chapter 2. Bacterial cells were grown in 100 mL R2A broth containing 2 mM of L-tryptophan and NaCl and PEG with intended concentrations (NaCl: 0.5%, 1% and 1.5%; PEG: 10% and 20%) and grown at 30 ºC and 130 rpm for 7 days. The pH of supernatants' pH was adjusted to 2.8. Then, ethyl acetate extraction and measurement of IAA content was performed by the same procedure as mentioned in Chapter 2.

ACC deaminase activity was assessed as described in Chapter 2. Bacterial cells were grown in R2A broth medium at 30°C, 130 rpm for 24 h. Then, cells were transferred to DF salt minimal medium (Dworkin & Foster, 1958) containing 3 mM ACC as the sole nitrogen source, and stressors at the intended concentrations were cultured at 30°C for 24 h. Then, cells were harvested and washed with 1 mL 0.1 M Tris-HCl (pH 7.6), resuspended with 0.1 M Tris-HCl (pH 8.5), and toluenized to release enzyme. ACC deaminase activity was evaluated by the capacity to convert an amount of 2,4-

dinitrophenyl-hydrazine to α-ketobutirate in 1 h. The protein concentration of cell extracts was determined by the Bradford protein assay (Bradford, 1976).

Biofilm production was measured as described in Chapter 3. Cells were grown in R2A broth containing NaCl and PEG in 96-well plates, incubated statically at 30°C for 24 h. In prior to incubation, the initial cell concentration was adjusted to be same in all conditions. Since cells were grown inadequately due to the presence of stressors, the amount of biofilm was expressed as the absorbance value (OD_{595}) of biofilm production divided by the absorbance value (OD_{595}) of bacterial growth.

4.2.3. Detection of tissue localization of HRRK170 in Chinese cabbage seedlings under stresses

Chinese cabbage seeds (Kigokoro 85 and 'Haregi 85) were surface-sterilized and inoculated with HRRK170 cells as described previously on 0.2% gellan gum (FUJIFILM Wako Chemicals, Japan) plates containing 0.2% HYPONeX, NaCl, or PEG 6000 at the intended concentrations. To prepare PEG containing plate, PEG was dissolved in 5 mM $MgSO_4 \cdot 7\ H_2O$ and 2.4 mM $CaCl_2 \cdot 2\ H_2O$ solution, while gellan gum and HYPONeX were dissolved in appropriate volume of distilled water. Then, two solutions were separately autoclaved and mixed only prior to plating inside clean bench. Plates containing NaCl were prepared in similar procedures that used for preparation of the PEG-containing plates. Inoculation and growth conditions were the same as described in Chapter 2. One week-old inoculated seedlings were separated from adhered semi-solid medium. Then, observation of the tissue localization of cells after GUS staining and determination of the infected cell density was performed as described previously in Chapter 3. Additionally, root segments from inoculated seedlings were excised from the maturation zone, treated with a LIVE/DEAD *Bac*Light bacterial Viability Kit (Invitrogen,

CA, USA) according to the manufacturer's instruction, and observed under fluorescence microscope.

To determine the localization of cells in roots of seedlings by SEM, roots were cut and fixed in 2% GA solution for 2 h, washed by adding 0.1 M phosphate buffer for 15 min three times, treated with 1% osmium tetroxide (OsO_4) for 2 h and washed with 0.1 M phosphate buffer for 15 min three times, followed by 50%, 70%, 80%, 90%, and 99% ethanol for 15 min, respectively. Then, samples were dehydrated with 99% tert-butyl alcohol, lyophilized, and coated with gold using an MSP-mini magnetron sputter prior to imaging on a SEM (Miniscope TM3030).

4.2.4. *Inoculation test of HRRK170 and plant growth promotion under stresses*

Seeds of Chinese cabbage (Kigokoro 85 and 'Haregi 85) were surface-sterilized with 70% ethanol and 1% sodium hypochlorite for 1 min, respectively, and rinsed with sterilized distilled water three times. Afterwards, seeds were sown on 1.5% agar plates in the dark for 3 days and seedlings were transferred to pot plug trays with PotAce N potting soil. Prior to transplantation, soil was sterilized at 70°C for 3 days and was inverted once to ensure proper sterilization. The soil was allowed to absorb sterilized distilled water containing NaCl and PEG with the intended concentrations until it was fully saturated with solutions to induce stress prior to the transplantation of seedlings. Inoculation procedures and growth condition were similar as described in Chapter 2. Plants growing in soil were watered by adding distilled sterilized water to the tray plate underneath the pot and allowing soil to absorb water and grown for 3 weeks. Plant above ground parts were weighed and after drying at 60 °C for 3 days, to obtain the fresh and dry weights, respectively. Effect of inoculation on plant growth was expressed as ratio (%) of the weights of inoculated plants against those of un-inoculated plants.

4.2.5. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows v.23.0 (IBM, Armonk, NY, USA). Data were subjected to the Student's *t*-test. A Tukey's honestly significant difference test with post-hoc comparison at the 5% confidence level was used to compare mean values among treatments. Experiments were performed at least twice.

4.3. Results

4.3.1. Salt and drought tolerance of HRRK170

The effects of NaCl- and PEG-induced stresses on the growth of HRRK170 cells were evaluated and result showed that HRRK170 grew in the presence of up to 1.5% NaCl or 20% PEG in R2A broth medium, although growth was retarded (Figure 13). In medium absence of NaCl, HRRK170 cells grew immediately; beside, in medium containing NaCl, the longer lag phase with increasing concentration of NaCl showed a slower adaptation. On contrary, bacteria rapidly adapted and grew in similar growth rate with control in a medium containing PEG. However, growth was retarded with increasing concentration of PEG in medium.

4.3.2. Effect of HRRK170 on growth of Chinese cabbage under stresses

Three growth-promoting compounds of HRRK170 were screened under stressed conditions (Table 3). Biofilm production per absorbance value of bacterial cells (OD_{595}) was increased by the presence of NaCl and PEG. When bacteria were exposed to high levels of salt stress (1.5% NaCl), the biofilm-producing capacity was increased approximately three-fold compared with the non-stressed condition (Table 3). High PEG concentrations (20%) in medium increased biofilm production; however, the effect was only1.3-fold (Table 3).

ACC-deaminase activity was assessed using DF medium containing ACC as the sole nitrogen source. This enzyme activity was not inhibited by the lowest concentration of NaCl (0.5%) but was inhibited by the higher concentrations of NaCl (1% and 1.5%). This trend was also observed in PEG affected conditions. Whereas the HRRK170 exhibited ACC-deaminase activity in the presence of 10% PEG, enzyme activity was inhibited by 20% PEG.

IAA production by the HRRK170 increased under salt and drought stressed conditions (Table 3). Salt improves bacterial IAA production up to 4.72 ± 0.03 µg mL-1 with 1% NaCl while the non-stressed condition resulted in IAA production of 3.71 ± 0.22 µg mL^{-1}. IAA production was the highest (8.67 ± 0.18 µg mL^{-1}) in medium containing 20% PEG.

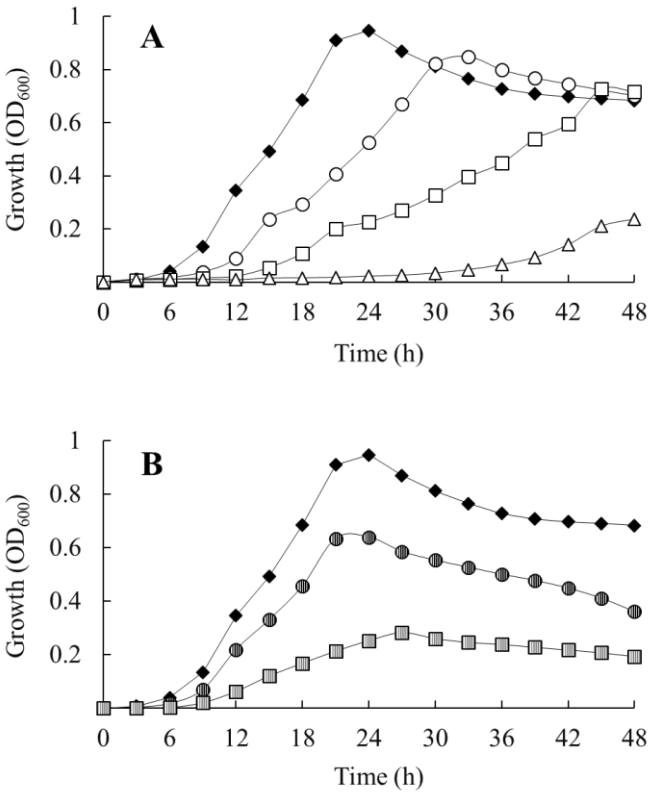

Figure 13. Growth of *Variovorax* sp. HRRK170 cells in presence of NaCl (A) and PEG (B) in medium. Symbols: ♦ Control (no stressors); ○, 0.5% NaCl; □, 1% NaCl; △,1.5% NaCl; ◍, 10％ PEG; ▥, 20% PEG. The experiment was performed three times, and representative result was shown.

Table 3. Biochemical activities of *Variovorax* sp. HRRK170 under salt and drought stresses

Stress	Concentration (%)	Biofilm (Biofilm A_{595}/Growth A_{595})	ACC deaminase (nmol α-ketobut. mg^{-1} protein h^{-1})	IAA (µg mL^{-1})
No stressors	-	0.78 ± 0.12^{a}	900.95 ± 25.76^{c}	3.71 ± 0.22^{a}
NaCl	0.5 %	1.86 ± 0.17^{c}	864.21 ± 33.78^{c}	4.55 ± 0.26^{b}
	1 %	2.19 ± 0.15^{d}	20.30 ± 2.12^{a}	4.72 ± 0.03^{b}
	1.5 %	2.42 ± 0.15^{d}	n.d.	3.78 ± 0.29^{a}
PEG	10 %	0.96 ± 0.13^{ab}	707.85 ± 18.90^{b}	6.63 ± 0.41^{c}
	20 %	1.08 ± 0.38^{b}	n.d.	8.67 ± 0.18^{d}

Values are mean ± SD. Data were compared by one-way analysis of variance. Values in the same column with common superscript letters are not significantly different ($p \leq 0.05$) by Tukey's honestly significant difference test. n.d.; not detected.

4.3.3. Plant tissue localization of HRRK170 in Chinese cabbage seedlings under stresses

When infected cell density was determined based on GUS-staining intensity, it was evidenced that HRRK170 comparatively densely (p<0.05) localized on plant root under higher concentrations of NaCl (1%, 1.5%) affected conditions (Figure 14). Also, high PEG concentration was also resulted slight increase in infected cell density, and effect was insignificant. Cell density infected to plant was not changed when plants grew in medium containing 0.5% NaCl and 10% NaCl as compared to control.

Plant tissue localization was visualized by GUS staining (Figure 15), bacterial viability kit (Figures 16 and 17) and SEM observation (Figures 18 and 19). When seedlings were grown on salt containing medium, primary and lateral root length were remarkably reduced with increasing concentration of NaCl (Figure 15). In terms of localization pattern, the colonization throughout the entire roots was observed in both plants, suggesting HRRK170 localize on plant in a similar trend in both stressors affected and normal condition.

Since Chinese cabbage seedlings have bushy root with dense root hair, observation using bacterial viability kit provided more detailed visualization of localization of HRRK170 and SEM observation provided cells on root. As it was showed on Figure 16, mostly HRRK170 cells localized in near of lateral root base of cv. Haregi 85 under salt stressed condition. However, this colonization was not common for cv. Kigokoro 85. Cells observed as it localized throughout main root of seedling in cv. Kigokoro 85 (Figure 17).

When inoculated seedling roots were observed by SEM, cells localized in a spread pattern on the root under non-stressors added condition (Figures 18 and 19). However,

mild stress induced cells aggregation and ultimately cells adhered thickly to each other with increasing salt concentration in agar. Drought stress induced by PEG also caused cell aggregation. However, the presence of 10% PEG resulted cells encased in layer of biofilms, while ticker and aggregated cells was evidenced under 20% of PEG induced stress as it was observed in high salt stressed condition. The evidence that high stress resulting cells aggregation led by biofilm was in meet the observation of bacterial viability of cells.

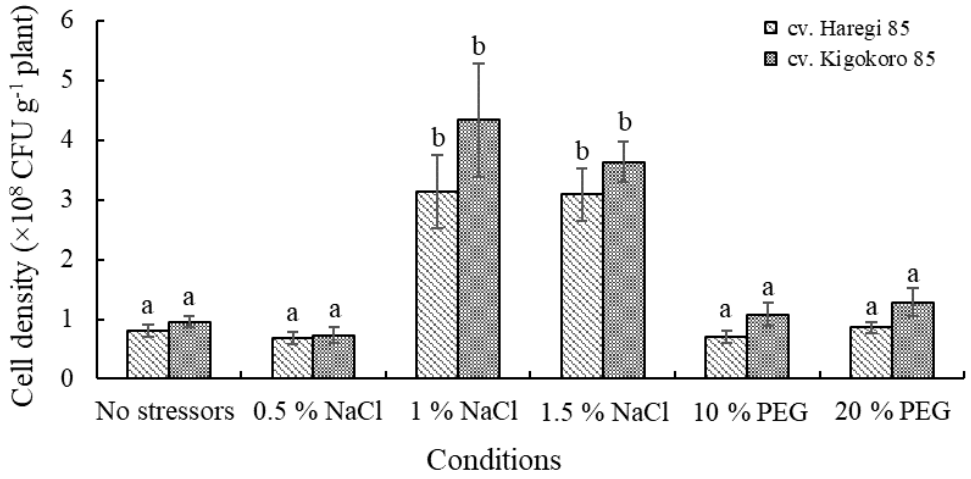

Figure 14. Cell density of *Variovorax* sp. HRRK170 infected in two cvr. of Chinese cabbage seedlings under salt and drought stresses. Cell density was calculated as the cell number per g of plant using the correlation curve described in Figure 10. Vertical bars show means ± standard deviations. Different letters above the bars indicate significant differences (p<0.05; Tukey's test) between means.

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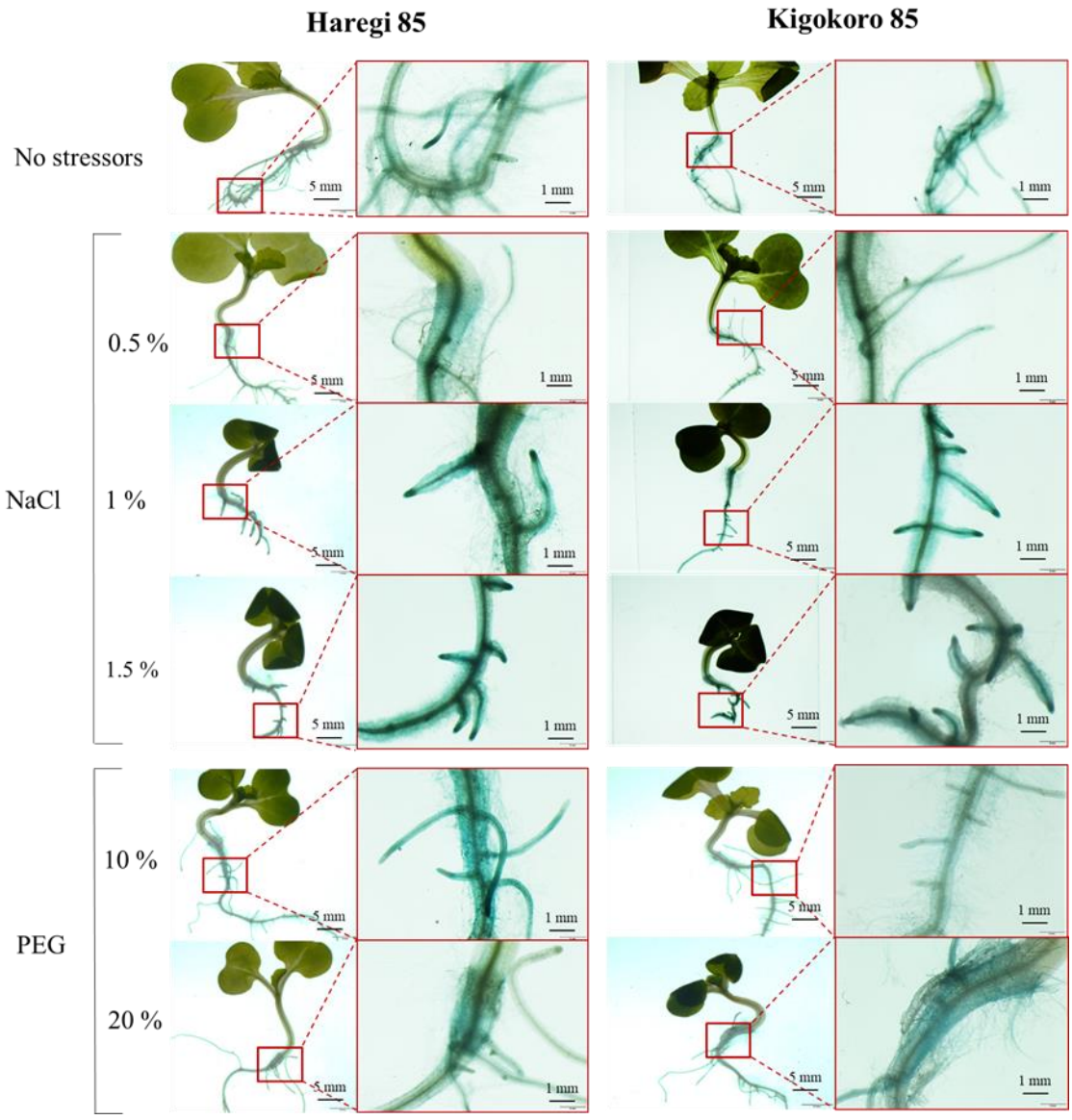

Figure 15. Tissue localization of GUS-stained *Variovorax* sp. HRRK170 in Chinese cabbage seedlings in the presence of NaCl or PEG. Enlarged box indicated the site HRRK170 localized area.

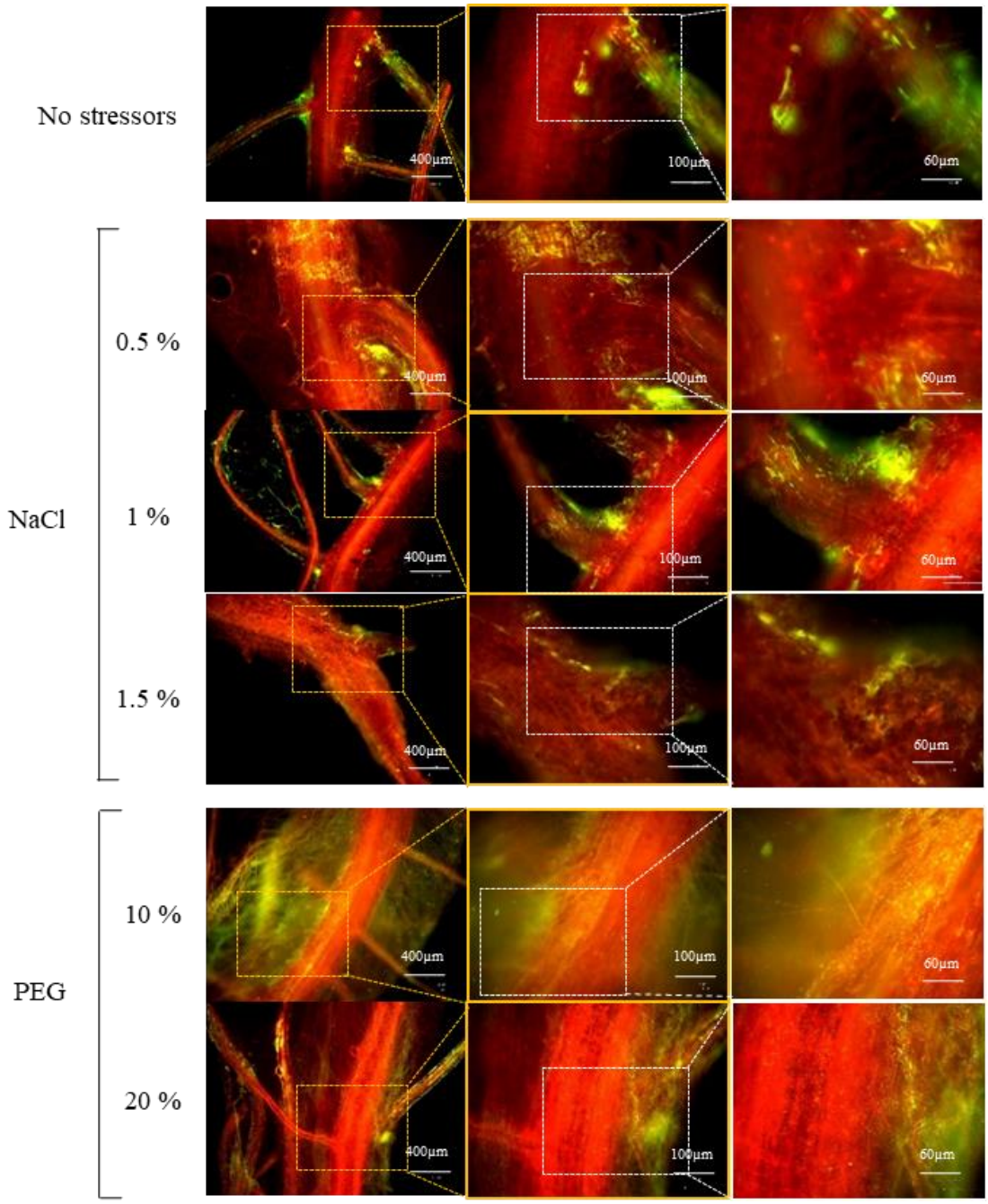

Figure 16. Bacterial viability of *Variovorax* sp. HRR170 cells localized in seedlings of Chinese cabbage cv. Haregi 85 in the presence of NaCl and PEG at 7 days of post-inoculation. Cells were stained with *Bac*light viability kit were observed under longpass filter. Enlarged box indicated the site HRRK170 localized area.

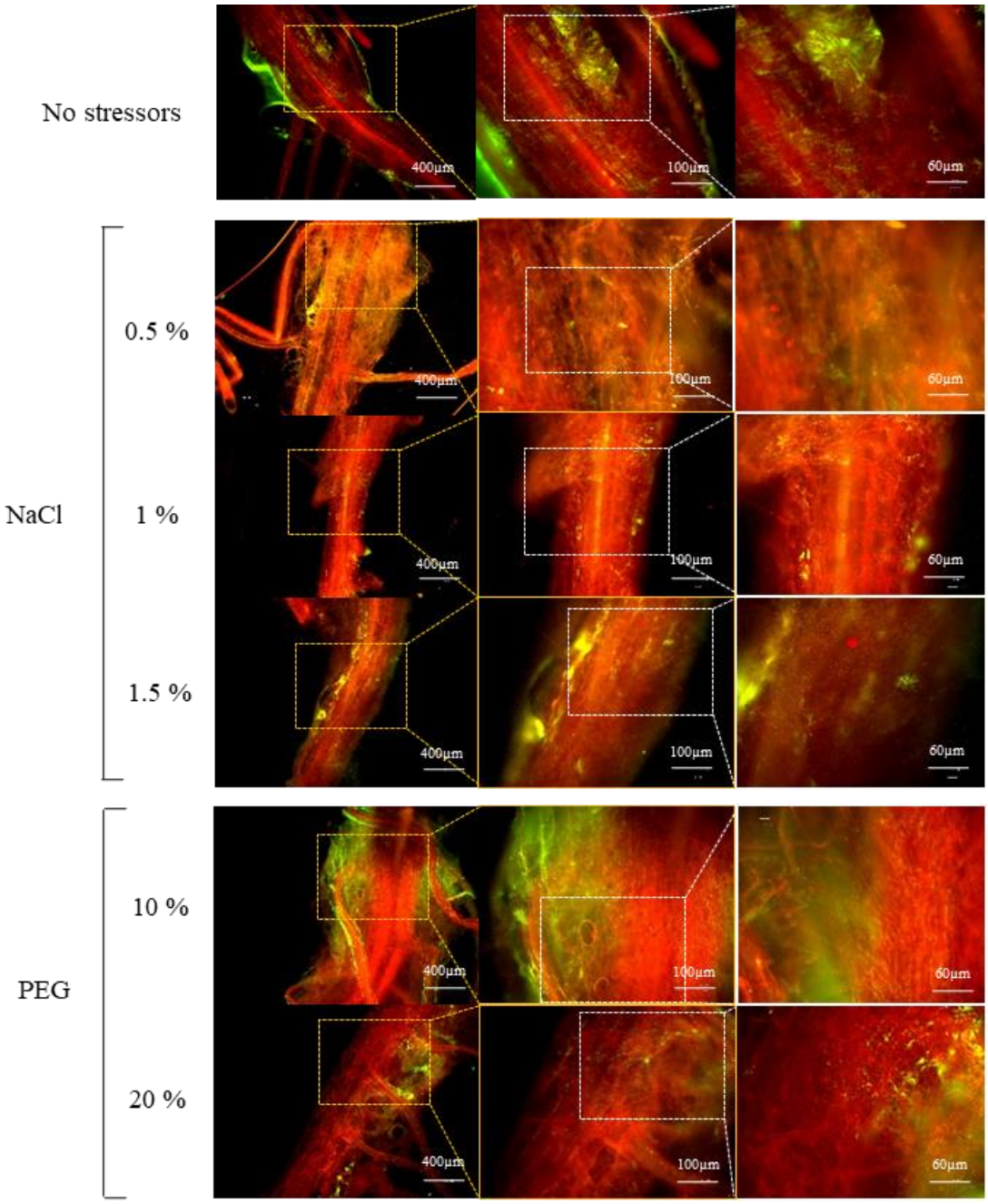

Figure 17. Bacterial viability of *Variovorax* sp. HRR170 cells localized in seedlings of cv. Kigokoro 85 in the presence of NaCl and PEG at 7 days of post-inoculation. Cells were stained with *Bac*light viability kit were observed under longpass filter. Enlarged box indicated the site HRRK170 localized area.

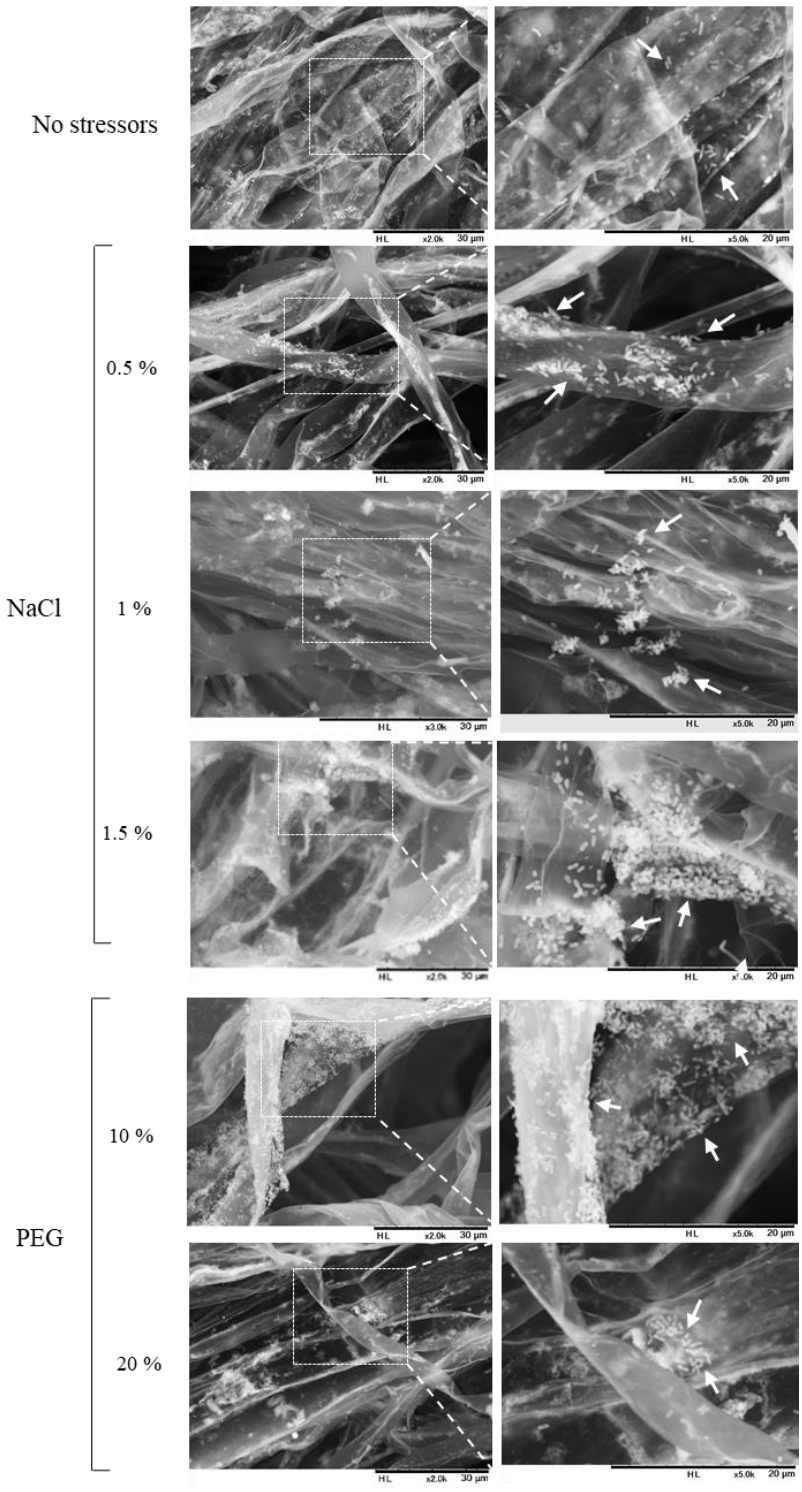

Figure 18. Scanning electron micrographs of localization of *Variovorax* sp. HRRK170 cells on seedlings of cv. Haregi 85 root grown in the presence of NaCl or PEG. Enlarged box indicated the densely colonized area of *Variovorax* sp. HRRK170 cells. Arrow indicated cells on the root.

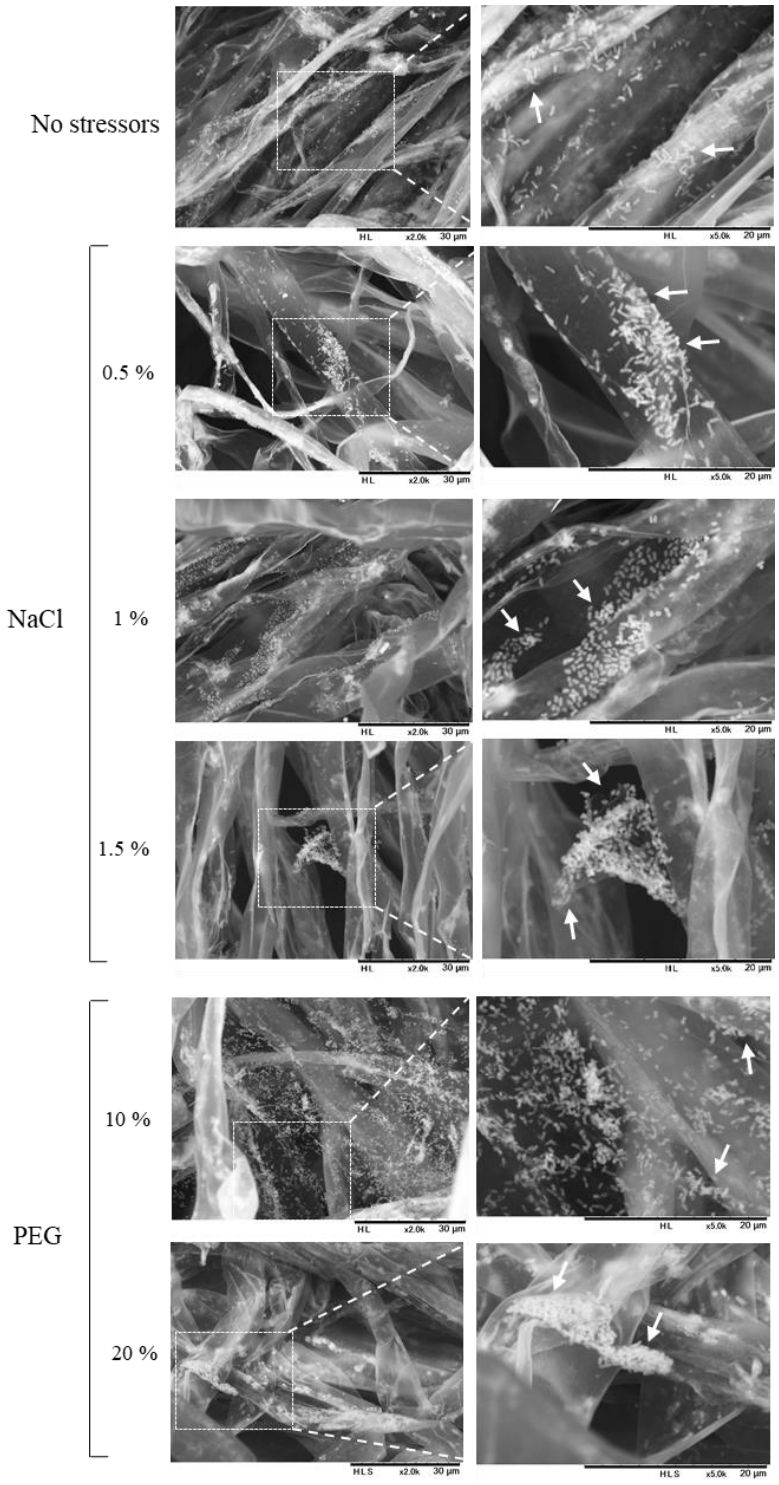

Figure 19. Scanning electron micrographs of localization of HRRK170 cells on seedlings of cv. Kigokoro 85 root grown in NaCl and PEG induced stressed conditions. Enlarged box and indicated the densely colonized area of HRRK170 cells. Arrow indicated cells on root.

4.3.4. Effect of HRRK170 on growth of Chinese cabbage seedlings under stresses

The stress-ameliorating effect of HRRK170 on Chinese cabbage growth is shown on Figure 20. The results show that the growth of cv. Kigokoro 85 was not stimulated by inoculation of HRRK170 while growth of cv. Haregi 85 was improved by inoculation of HRRK170 under normal conditions. Although inoculation of HRRK170 stimulated growth of both plants under mild salt stresses (0.5% and 1%NaCl), but not significantly (p=0.005), it exerted significant growth promotion (p>0.05) in both plants under high salt stress (1.5%), compared with the uninoculated control (ratio (%) of the weights of inoculated plants against those of uninoculated plants: cv. Haregi 85, 135.7 and 155.9; Kigokoro 85, 178.6; Kigokoro 85, and 178.6 and 175.4, in terms of fresh and dry weights, respectively).

Drought stress induced by PEG showed different effects on plants growth. Whereas, growth of Kigokoro 85 was significantly improved by159.2 % and 125.2 % , growth of cv. Haregi 85 was not significantly improved under 20 % PEG induced stress. Although the survival of both plants reduced under high drought stress, cv. Haregi 85 rapidly recovered and subsequently grew, indicating that the it was tolerant against stress.

Overall, the HRRK170 acted to mitigate salt stress in both two cultivars of Chinese cabbage, and this effect was more clearly evidenced under high stress. In particular, drought stress was mitigated by the presence of HRRK170 cells in cv. Kigokoro 85, which appeared to be more susceptible to stress than cv. Haregi 85.

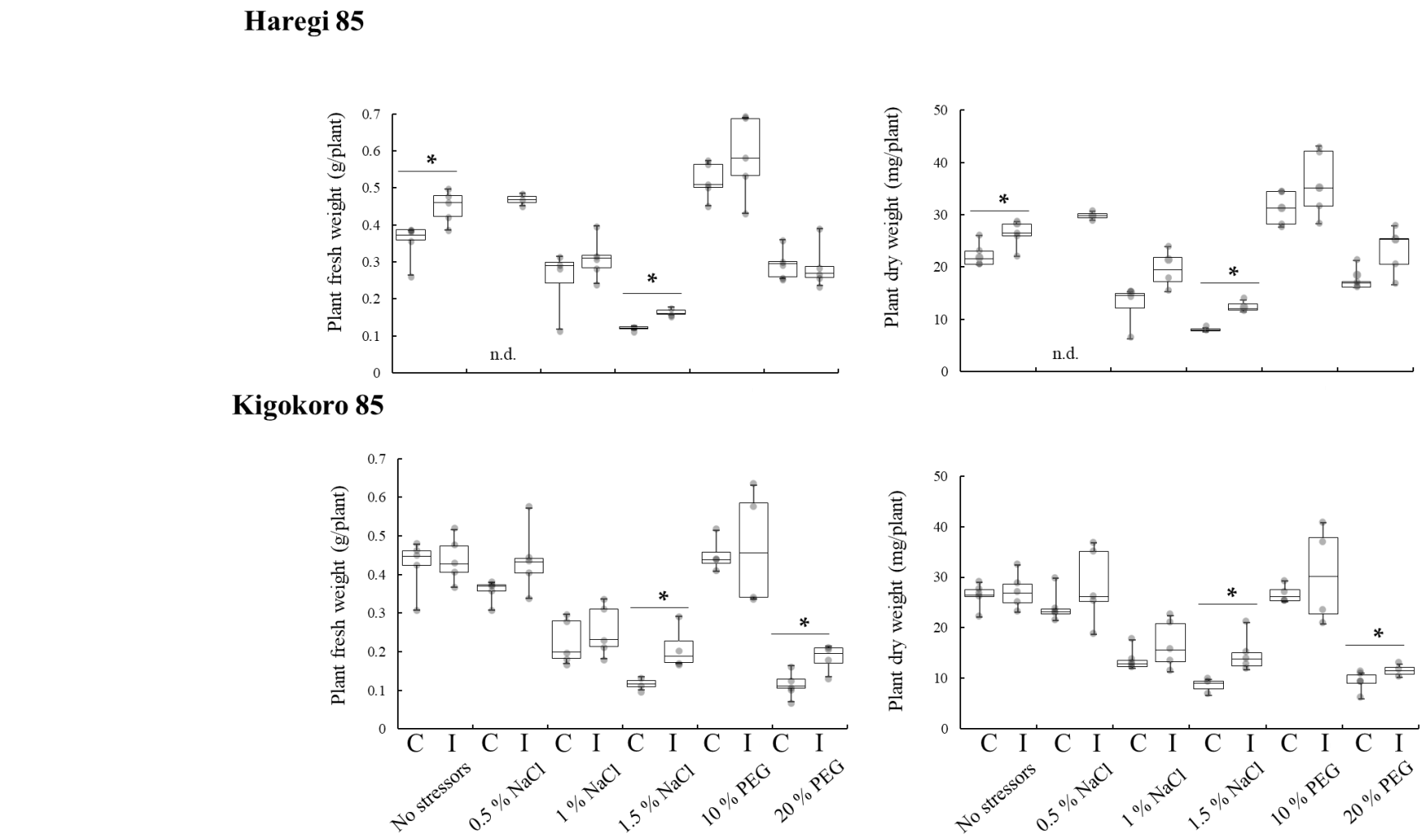

Figure 20. Effect of *Variovorax* sp. HRRK170 on the growth of Chinese cabbage 'Haregi 85' (A) and 'Kigokoro 85' (B) under salt or drought stressed condition. Plant fresh weights and dry weights are shown as means ± standard deviation. Mean values were compared by *t*-test. Asterisks indicate significant (*, $p \leq 0.05$) differences between inoculated (I) and un-inoculated control (C). n.d. : not determined.

4.4. Discussion

To understand the effects of *Variovorax* sp. HRRK170 as plant stress mitigating bacteria, its biochemical characteristics, stress tolerance as well as inoculated plant's growth response along with bacterial plant tissue localization had been investigated under salt and drought stresses. Since 1.5 % NaCl and 20 % PEG were adequate to induce stress in both plant and bacteria, causing retarded growth, these conditions were selected to evaluate the effect of HRRK170 on plant growth. Additionally, the growth response of plants to mild salt and drought stress (0.5 and 1% NaCl, and 10 % PEG) was also investigated.

HRRK170 also produced increased amount of IAA under salt and drought stress (Table 3). The production of phytohormones, particularly IAA, is important for promoting plant growth (Glick, 1995). Increasing IAA production (up to a three-fold) has been reported on *Bacillus megaterium* and *Pseudomonas putida* by adding PEG (up to 60%) to the medium (Marulanda *et al.*, 2009). In general, increased IAA production of HRRK170 represents bacterial adaptation to survive under stressed condition by promoting plant growth. In a previous study, PGPB was shown to improve the growth of Chinese cabbage under stressed conditions (Lee *et al.*, 2018). Particularly, IAA acts to increase root/root hair length or proliferation, which can improve water availability in roots (Timmusk, Abd El-Daim, Copolovici, Tanilas, & Kännaste, 2014), (Timmusk *et al.*, 2014), especially for shallow-rooted plants such as Chinese cabbage (Murakami, Yamada, & Yoshida, 2002). Under salt stress, increased seed germination and seedlings growth were observed in Chinese cabbage inoculated by IAA-producing PGPB (Hussein, Yoo, & Joo, 2016). Therefore, IAA production by the HRRK170 might act to stimulate Chinese cabbage growth in such condition.

Also, the results showed that HRRK170 was able to produce biofilm under the given conditions. Biofilm production is an one bacterial strategy for survival following exposure to stress (Ansari & Ahmad, 2018). Generally, bacterial biofilms are essential for protecting against desiccation, microbial aggregation, plant microbe interactions, and surface attachment (Bogino, de las Mercedes Oliva, Sorroche, & Giordano, 2013). PGPB bacterial biofilms also help plants to improve the utilization of water in soil that lead improved growth and increased survivability (Kasim, Gaafar, Abou-Ali, Omar, & Hewait, 2016) by reducing the harmful effects of salt stress on plants through sequestering Na^+ into vacuoles and expelling it from roots (Chen *et al.*, 2016) and ultimately increseing water availability in roots under drought stress (Timmusk *et al.*, 2014). Salt mitigation by biofilm-producing PGPB has previously been reported for chickpea (Qurashi & Sabri, 2012) wheat (Timmusk *et al*., 2014) barley (Kasim *et al*., 2016) and faba bean (Mohammed, 2018).

When the density of infected cells was investigated by GUS staining, the results indicated that HRRK170 localized on seedlings roots at higher levels under 1% and 1.5% NaCl affected conditions (Figure 15); these results are somewhat consistent, but not correlated, with the increased biofilm production by HRRK170 cells under stressed conditions (Table 3). Presumably, infected cell density to plants was partially determined by biofilm production that may increase attachment to surface of plant. Additionally, infected cell density might be influenced by other factors such as plant growth response to given conditions. In general, infected cell density was lower in all conditions than the result mentioned in chapter 4, suggesting cell density localized on plant can fluctuate depending on growth condition. The tissue localization of HRRK170 cells also demonstrated that aggregated bacterial cells in biofilm attached to the surface of the root (Figure 18, 19), which was not observed under normal conditions.

A pot trial revealed that the HRRK170 mitigated stresses, particularly high stresses (Figure 20). Tolerance against high salt (1.5% NaCl) stress was improved by inoculation in both cv. Haregi 85, in which growth was stimulated under non- stressed conditions, and in cv. Kigokoro 85, which was not responsive to HRRK170 under normal conditions. Although the survival of plants was reduced by 20% PEG, cv. Haregi 85 rapidly recovered and subsequently grew, indicating that the cultivar was tolerant against stress. The highest growth promotion tended to occur under high stress

Collectively, the results of this study indicated that HRRK170 could mitigate salt and drought stresses in plant and results encourage further studies on the use of HRRK170 as a stress mitigating.

CHAPTER 5.

Summary, conclusions and recommendations

Sustainable agricultural production through environmentally friendly approaches is the most effective way to meet increasing food demands worldwide. One promising candidate is the application of microbes, which is co-evolutionary partner of plants. Application of plant growth-promoting bacteria (PGPB) that form a symbiotic relationship with plants can enhance yield and improve plant tolerance against stresses. Plant-microbe interactions are complex and dynamic and affected by various factors. Better understanding about effectual role of PGPB to host plant growth can contribute to develop microbial approach that can be successfully used for sustainable agriculture. Therefore, it is important to investigate growth promoting profiles of beneficial microbes for its effectual application.

Sugar beet (*Beta vulgaris* L.) is an essential sugar producing crop. Owing to its diverse bacterial community with excellent tolerance against local environmental conditions, this plant has gained attention as a study object. We anticipated that bacterial strain isolated from the sugar beet may also increase the growth of other plants such as vegetables. Vegetables are more sensitive than other horticultural plants to abiotic stresses, while it is an important part of the human dietary as it is rich in many essential vitamins, carbohydrates, salts and proteins. Application of PGPB in vegetables production system improves yield due to improved root length, stress tolerance, seed ageing and germination, freshness, as well as nutritional value and properties.

Previously, bacterial strains having a high affinity with the sugar beet (*Beta vulgaris* L. cv. Rycka) were examined for their plant growth-promotive abilities (Kenkyuseika, vol. 539. 2015. Tsukuba Office, Agriculture, Forestry and Fisheries Research Council Secretariat, Japan). This study aimed to identify the most potential strain among bacterial isolates as bioinoculant with regard to its PGPB traits and evaluate the selected strain's effectiveness on growth of vegetable seedlings, including cabbage (*Brassica oleracea* L. cv. Harunami), lettuce (*Lactuca sativa* L. cv. Cisco), tomato (*Solanum lycopersicum* L. cv. Momotaro), radish (*Raphanus raphanistrum* L. cv. Taibyo sobutori), eggplant (*Solanum melongena* L. cv. Senryo no.2), Chinese cabbage (*Brassica rapa* L. cv. Kigokoro 85, Kigokoro 65, Haregi 85, and Okiniiri), and green pepper (*Capsicum annuum* L. cv. Kyomidori, Kyonami, Ace, and Pitaro)].

Six plant growth-promoting bacterial strains (*Rhizobium* sp. HRRK 005, *Polaromonas* sp. HRRK103, *Variovorax* sp. HRRK170, *Mesorhizobium* sp. HRRK 190, *Streptomyces* sp. HRTK192, and *Novosphingobium* sp. HRRK193) isolated from sugar beet (*Beta vulgaris* L.) were screened using a series of biochemical tests. Production of IAA was quantified by HPLC analysis using ethyl acetate extraction method (Tien, Gaskins, & Hubbell, 1979), siderophore production was evaluated using chrome azurol S shuttle assay (Schwyn & Neilands, 1987) and biofilm production was determined by microtiter plate assay (Yuttavanichakul *et al.*, 2012). Also bacterial growth under wide range of temperature and pH was investigated. Among all strains screened, HRRK170 strain had the highest potential for plant growth promotion, given its ability to produce plant growth substances and enzymes such as siderophores and ACC-deaminase, respectively, concomitantly with active growth in a wider range of temperatures (10–30 ^{o}C) and pH values (5.0–10.0). Its epiphytic infection profile to sugar beet seedling root

was confirmed by SEM or light microscopic observation through inoculation of GUS-labeled cells on 0.3 % agar plate containing 2000-fold dilution of HYPONeX 6-10-5.

Moreover, HRRK170 colonized either as spots or widely on the plant root surface including root hair of all vegetable seedlings tested, but significant growth promotion occurred only in two vegetables (Chinese cabbage and green pepper) as well as the sugar beet, and also showed an upward tendency for two vegetable seedlings (cabbage and eggplant). However, HRRK170 failed to function as a plant growth promoter for the other three vegetables (tomato, radish, and lettuce). The cell density of HRRK170 localized in the plant roots (cell number per g of plant) could be estimated using correlation curve we designated, because most of the cells localized on the epidermis. When the density of infected cells on seedlings was determined based on GUS-staining intensity, the cell density varied depending on plant species and cultivars. Furthermore, results showed that optimally infected cells led significant increment in plant growth, as observed in green pepper 'cv. Kyonami' and Chinese cabbage 'cv Haregi 85' through inoculation tests using pot soil. Therefore, it was assumed that the optimum cell density may reflect the beneficial interaction between plants and HRRK170 strain.

Additionally, salt and drought stresses (induced by NaCl and PEG) mitigating effect of HRRK170 was investigated using two cvr. of Chinese cabbage of which growth was significantly stimulated (Haregi 85) or not effected (Kigokoro 85) by the presence of HRRK170 cells under normal condition. The efficient and stable localization of HRRK170 cells on roots that grown under stressed conditions was confirmed by SEM observations staining of GUS-labeled cells. Furthermore, increased tolerance against high salt (1.5 % NaCl) was recorded on both plants, while drought stress (20 % PEG) was mitigated only in cultivar Kigokoro 85, which seemed more sensitive to stresses than the other one. In parallel, the biochemical profile of this strain was screened under stressed

conditions. The results suggested that the contribution of this strain to plant stress tolerance was reflected by its improved IAA and biofilm production under stressed conditions. Biofilm production by HRRK170 might not only provide stable attachment of cells on plant root, but also minimize ion toxicity when plant was exposed to salt stress. Bacterial increased IAA production under drought stress also could play in role in stimulated plant growth. Therefore, HRRK170 acted as salt and drought stress mitigating bacteria in plants with non-host specific characteristic, and effect was more clear on growth of plant that seemed susceptible to these stresses.

In conclusion, this study showed that HRRK170 strain isolated from sugar beet is the most potential bioinoculant and plant stress mitigating bacteria for having higher tolerance against wider temperature range and pH and multiple advantageous biochemical characteristics. Also, HRRK170 displayed efficient infection profile as it epiphytically associated with all vegetables seedlings root. However, its infecting cell density to plant is an important factor in determining whether or not the bacterial strain fully functions to promote plant growth. Additionally, this strain showed plant stress mitigating bacteria, when host plant was undergone to high salt (1.5 % NaCl) and drought (20 % PEG). Therefore, HRRK170 strain could be used as a plant growth promoter with optimum infecting cell density and plant salt and drought stress mitigating bacteria. Findings of this study may provide better understanding about HRRK170 strain's role in plant growth and contribute further to studies on HRRK170 strain for future application.

Summary (in Japanese)

世界中で増加する食料需要を満たすため、植物生産システムが安定で高収率に最適化されているが、農薬を使用しない環境調和型の持続可能な農業生産は、その最も効果的な方法である。植物に共生する微生物の利用は、有望な候補のひとつである。植物との共生関係を形成する有益な微生物の利用は、収量を増強し、ストレスに対する植物の耐性を改善することが可能である。植物と微生物の相互作用は複雑で動的であり、さまざまな要因の影響を受ける。植物の成長を促進する Plant growth promoting bacteria (PGPB)の役割についての理解は、持続可能な微生物利用法を開発することに貢献する。したがって、有益な微生物の成長促進プロファイルを調査することは、それらの利用のために重要である。

テンサイ（*Beta vulgaris* L.）は、砂糖生産における主要作物である。局所的な環境ストレスおよび土壌条件に対する優れた耐性を示す多様な細菌群集を持つため、この植物は研究対象として注目を集めている。テンサイから単離された細菌株も野菜などの他の植物の成長を促進する可能性があると予想された。テンサイとは異なり、野菜は他の植物よりも非生物的ストレスに敏感だが、多くの必須ビタミン、炭水化物、塩、タンパク質が豊富に含まれているため人間の食料として重要である。野菜生産システムにおける PGPB の利用によって、根の伸長、ストレス耐性、種子の発芽、鮮度、ならびに栄養価が強化され収量を増大させる。

以前、テンサイ（*Beta vulgaris* L.）と高い親和性を有する細菌株の植物生育促進能力について調べた。本研究は、PGPB として最も有望な細菌株を同

定し、キャベツ（*Brassica oleracea* L. cv. Harunami）、レタス（*Lactuca sativa* L. cv. Cisco）、トマト（*Solanum lycopersicum* L. cv. Momotaro）、ダイコン（*Raphanus raphanistrum* L. cv. Taibyo sobutori）、ナス（*Solanum melongena* L. cv. Senryo no.2）、ハクサイ（*Brassica rapa* L. cv. Kigokoro 85, Kigokoro 65, Haregi 85, and Okiniiri）、ピーマン（*Capsicum annuum* L. cv. Kyomidori, Kyonami, Ace, and Pitaro）の植物実生への成長に対する有効性を評価することを目的とした。

テンサイから単離された 6 種の植物生育促進細菌株（*Rhizobium* sp. HRRK005、*Polaromonas* sp. HRRK103、*Variovorax* sp. HRRK170、*Mesorhizobium* sp. HRRK190、*Streptomyces* sp. HRTK192、および *Novosphingobium* sp. HRRK193）を、いくつかの生化学的試験を用いてスクリーニングした。インドール－3－酢酸（IAA）の生成は、酢酸エチル抽出法を用いた高速液体クロマトグラフィー（HPLC）分析によって定量した(Tien, Gaskins, & Hubbell, 1979)。シデロフォア産生を Chrome azurol S shuttle 分析(Schwyn & Neilands, 1987)を用いて評価し、バイオフィルム産生をマイクロタイタープレートアッセイ(Yuttavanichakul *et al.*, 2012)により決定した。

広範囲の温度および pH 条件下での細菌増殖も調べた。HRRK170 株は、広い温度範囲（10～30℃）と pH（5.0～10.0）において植物成長物質ならびに酵素、例えばシデロフォアおよび 1-アミノシクロプロパン-1-カルボン酸（ACC）デアミナーゼのいずれの同時生産が可能であったことから、スクリーニングされた株の中で最も植物成長促進の可能性が高いと思われた。テンサイの根への感染プロファイルは、2000 倍希釈された HYPONeX®を含む 0.3%寒天プレートへ

のGUS標識細胞の接種による走査型電子顕微鏡（SEM）または光学顕微鏡によって観察された。

HRRK170 は、すべての植物体で斑点状、または広く根の表面に定着したが、有意な生長促進は、植物根に局在する特定の細胞密度範囲内の 2 つの野菜（ハクサイおよびピーマン）においてのみ確認された。 HRRK170 の接種は 2 つの野菜（キャベツおよびナス）で増加傾向を示したが、他の 3 つの野菜（トマト、大根、レタス）では植物生育促進剤として機能しなかった。ほとんどの菌は表皮に局在しており、植物の根に局在する HRRK170 の細胞密度（植物 1 g 当たりの細胞数）は、作成した相関曲線を使用して推定することが可能であった。感染細胞密度を GUS 染色強度に基づいて決定したところ、植物種および栽培品種によって異なっていた。さらに、ポット土壌を用いた接種試験において、ピーマンの栽培品種 Kyonami とハクサイ Haregi 85 は、最適な細胞数で感染した植物は成長を有意に増加させることが示された。したがって、HRRK170 の最適菌密度は植物との間に有益な作用を示すと考えられた。

さらに、NaCl および PEG によって誘導される塩分および乾燥ストレスを緩和する効果を、成長が有意に促進された Haregi 85、または影響を受けない Kigokoro 85 の白菜 2 品種を用いて調べた。ストレス条件下で増殖した根における GUS 標識 HRRK170 の局在化は、染色と SEM 観察によって確認された。さらに、高塩濃度（1.5%NaCl）に対する耐性の増加は 2 つの栽培品種の両方でみられたが、乾燥ストレス（20%PEG）は Kigokoro 85 のみ軽減された。同時に、この菌株の生化学的プロファイルをストレス条件下でスクリーニングした。その結果、この株がストレス条件下で IAA およびバイオフィルム生産の増大し、植

物ストレス耐性が向上することが示唆された。 HRRK170 によるバイオフィルム生産は、植物の根細胞への安定した付着だけでなく、植物が塩ストレスに曝されたときのイオン毒性を最小限に抑えた。また、乾燥ストレス下で細菌の増加した IAA 生産も植物成長を促進した可能性がある。このように、HRRK170 は、宿主特異性をもたない植物において、塩分および乾燥ストレスの軽減作用を有し、これらのストレスに影響される植物の成長に明らかに作用していた。

研究結果は、テンサイから単離された HRRK170 がより広い温度範囲および pH に対する耐性と優れた生化学的特性を有するため、有望な微生物資材および植物ストレス軽減剤であると結論付けられた。また、HRRK170 はすべての植物体と効率的な感染がみられた。植物体への感染細胞密度は、細菌株が植物成長を促進するために十分に機能するかどうかを決定する重要な因子である。植物は高塩濃度（1.5%NaCl）および乾燥（20%PEG）条件下でストレス緩和効果を示した。したがって、HRRK170 株は最適な細胞密度で植物に感染させることによって、塩および乾燥ストレス軽減効果を持つ植物成長促進剤として使用することが可能であった。 この研究の結果は、植物成長における HRRK170 株の役割についての知見を提供し、HRRK170 株の今後の実用化のためのさらなる研究に貢献するかもしれない。

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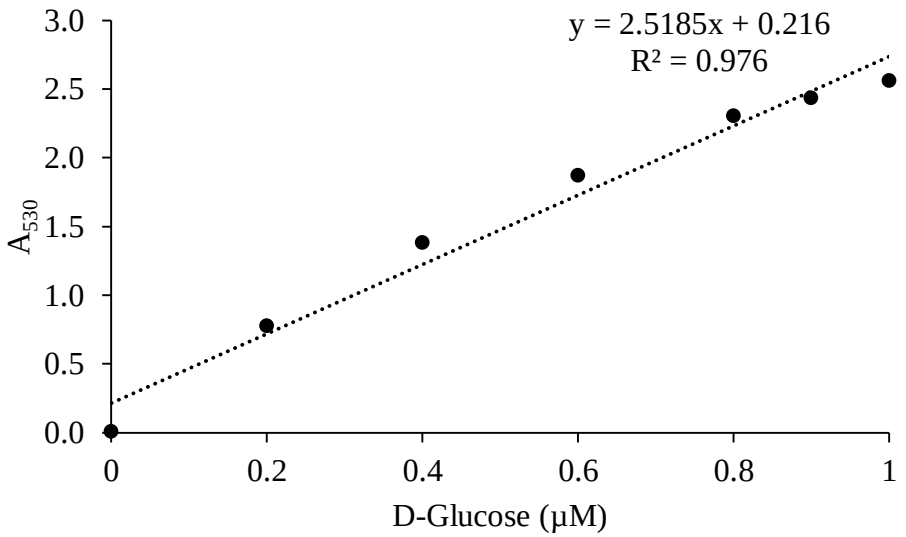
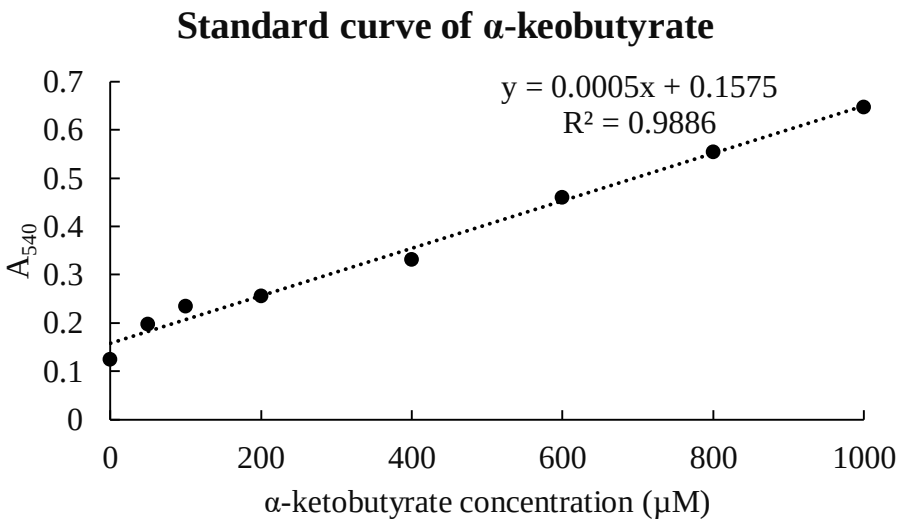
APPENDICIES

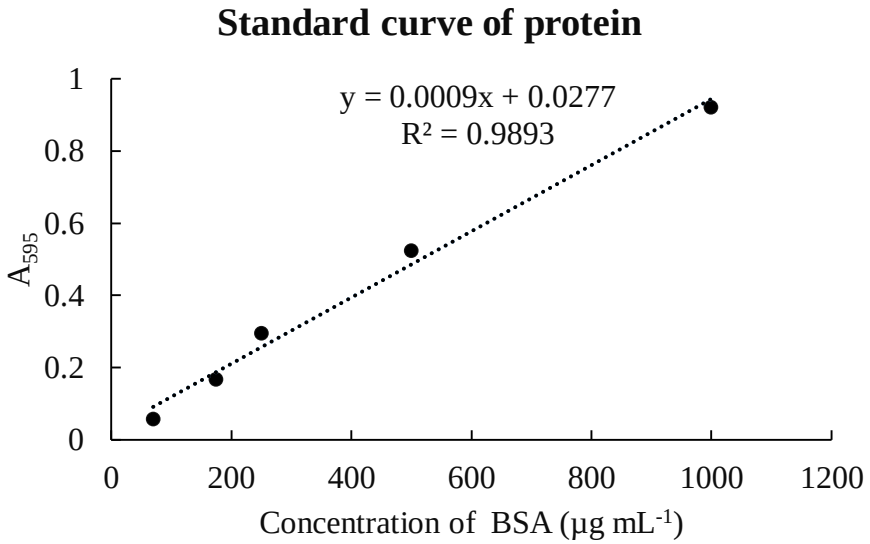
Starndard curve for β-glucanase



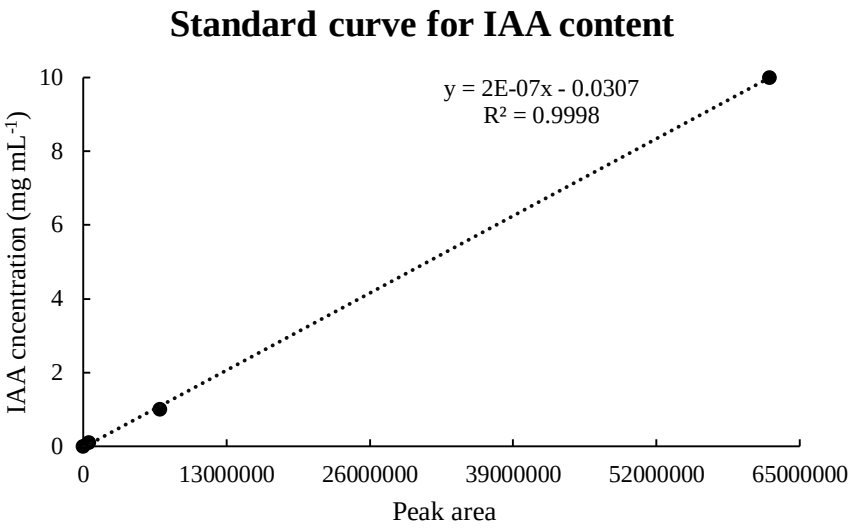
Appendix 1. Standard curve for D-glucose. Vetical axes show absorbance value of standard solution at different concentration of glucose. B-glucanase activity was determined by computed following the linear equation (y=mx+b) displayed in the chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.



Appendix 2. Starndard curve for α-ketobutyrate. Vertical axes show absorbance value of standard solution at different concentrations of α-ketobutyrate. ACC deaminase activity (x) was computed following the linear equation (y=mx+b) displayed in the chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.



Appendix 3. Standard curve for protein. Vertical axes show absorbance values of standard solution at different concentrations of Bovine serum albumin. Protein concentration was determined by computing with following linear equation (y=mx+b) displayed in chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.



Appendix 4. Standard curve for IAA. Vertical axes show absorbance values of standard solution at different concentrations of IAA. IAA concentration was determined by computing with following linear equation (y=mx+b) displayed in chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.